Apoptosis and Keloid Scarring:

Potential for Laser Interaction

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Abstract

Keloid scarring is an extremely challenging form of abnormal scarring and the underlying biological mechanisms in its pathogenesis have not been established. There are multiple existing therapeutic approaches but all have a varying response with none showing a consistent advantage. Keloid-derived fibroblasts are observed to exhibit resistance to apoptosis during the course of scar maturation. In order to gain an insight into the pathological apoptotic regulation of keloids scars, 3 pairs of normal skin and keloid scar fibroblasts derived from tissue samples collected from the same patients were analyzed for significant difference in the expression of apoptotic related genes using microarray. The microarray results were then confirmed using real time RT-PCR analysis. In this study, 5 genes were identified to be differently expressed in normal skin and keloid scar derived fibroblast, namely DcR3, Fn14, TNFR2, DcR2 and OPG. These genes all play crucial roles in the regulations of wound healing and scar formation. In addition, the treatment modality that can potentially modulate the apoptosis regulation in keloid was also investigated in the study. The Nd:YAG 532 nm laser is an emerging modality for the treatment of keloid scarring. Previous studies by our group have shown that the QS 532 nm Nd:YAG laser is able to regulate gene expressions in normal fibroblast cultures. Further to this observation, the biological effects of Nd:YAG 532nm laser radiation on cell death and proliferation in keloid-derived fibroblasts were investigated in this study. Monolayer cell cultures of keloid scar fibroblasts were irradiated with the QS 532 nm Nd:YAG laser with different energy. Irradiated cells were analyzed for changes in cell viability, proliferation and apoptosis at 24 and 48 hours after laser treatment. The results showed that the QS 532 nm Nd:YAG laser can induce apoptosis in keloid-derived fibroblasts and the percentage of necrotic cell death was relatively low. It indicated that this laser may potentially induce apoptosis within a keloid lesion and trigger only minimal inflammatory response, and hence produce a lower chance of recurrence.

瘢痕疙瘩的成因一直未被醫學界確定，使其治療變得極具挑戰性。迄今爲止，只有很少數瘢痕疙瘩的個案能夠被成功治癒。觀其原因，可能在創傷後修復的過程中，瘢痕疙瘩裡的成纖維細胞比正常的成纖維細胞更能抵抗細胞凋亡，導致組織異常增生。爲了更加清楚了解細胞凋亡和瘢痕疙瘩的病理關係，我們在是次研究中收集了來自三位患者的正常皮膚成纖維細胞和瘢痕疙瘩成纖維細胞，進行針對細胞凋亡基因的陣列分析（microarrays），從而篩選出在基因陣列中表現出顯著差異的基因，當中包括 DcR3，Fn14，TNFR2，DcR2 和 OPG，並且以 real-time PCR 進行驗證。文獻指出這些基因都在創傷癒合和瘢痕疙瘩形成的過程中發揮著關鍵的作用。除此之外，是次研究亦嘗試了解現有的治療方法對成纖維細胞的細胞凋亡過程的影響。波長 532ｎｍ 的 Nd：YAG 激光輻照是一種治療瘢痕疙瘩的新療法。跟據本研究小組過往的研究顯示，波長 532 nm 的 Nd：YAG 激光有改變正常成纖維細胞的基因表達的能力，所以在是次研究中我們嘗試更詳細剖析 Nd：YAG　532 nm 激光輻照對瘢痕疙瘩的成纖維細胞的影響。爲了對比成纖維細胞的生長，壞死以及凋亡在激光輻照前後的變化，我們對瘢痕疙瘩的成纖維細胞施行不同能量的 QS 532 nm Nd：YAG 激光輻照。結果顯示，在輻照後的４８小時內，Nd：YAG532 nm 的激光可有效誘導瘢痕疙瘩的成纖維細胞凋亡而並非壞死。這意味著，Nd：YAG532 nm 的激光有可能是一種低炎症反應的治療方法，從而減低瘢痕疙瘩復發的機會。

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Dedication

I dedicate this thesis to my wonderful parents, who offered me unconditional love and support. Throughout the course of this study, they have been a great source of motivation and encouragement.

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1 Introduction to Keloid Scarring – The Pathogenesis and Treatment

1.1 The Pathogenesis of Keloid Scarring

The term keloid was first used by Alibert in 1806, and is derived from a Greek word meaning the "crab claw", which shows a resemblance of the scar being red, raised and with protrusions extending sideways around the boundary. Keloid scarring is unique to the human race, and this makes keloid research particularly difficult due to the lack of animal models (Kelly, 2009). Keloid is characterized by a scar that proliferates beyond the margins of the wound. Clinically, keloid is defined as a raised, erythematous, pruritic, fibrous lesion that will grow beyond the original boundary of the wound. This is also the most important clinical feature of keloid. While normal scar will mature gradually, appearing paler, softer and decrease in the scar volume, keloid scar on the contrary, will proliferate and remain active for a prolonged period of time and does not regress. Patients often report itchiness or pain in active Keloids. Therefore, keloid scarring is often described as a benign dermal fibroproliferative tumor, however with no malignant potential (Butler et al, 2008).

Keloid scars are most commonly found on the chest, shoulders, the upper back, the neck and the earlobe. There is an increasing incidence with an increase in racially defined pigmentation (Brown et al, 2008; Marneros et al, 2001). Keloid is observed usually following injury, such as acne, vaccination, burns, surgical wound healing and lacerations etc, and it commonly develops months to even a year after the injury (Juckett et al, 2009). Despite the research efforts of numerous scientists across the world, the aetiology of the keloid remains unclear. The mechanism of the development appears to be a result of an abnormal wound healing process, which leads to the fibroproliferative disorder. This may include the imbalance between degradation and production of the extracellular matrix, abnormal cell migration, proliferation and elimination (Seifert and Mrowietz, 2009).

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Wound healing is a very complex, yet carefully regulated process that involves a large number of cells and molecules. When injured, the release of intracellular factors into the extracellular fluid will activate the surveillance inflammatory cells to trigger the immune response. In the early phase of wound healing, the inflammatory cells will release pro-inflammatory cytokines and growth factors to induce angiogenesis and hence increase blood supply to support tissue repair. These cytokines will also activate

fibroblast proliferation and increase synthesis of collagen for the formation of granulation tissues, which will eventually develop into a scar (Velnar et al, 2009).

When the inflammation response subsides, active inflammatory cells will be removed mainly via apoptosis, an important pathway used to remove unwanted cells by letting them "commit suicide" (Rai et al, 2005). The level of cytokines and growth factors will hence return to the basal level. To avoid excessive production of scar tissue, endothelial cells that formed the small blood vessels will be removed to reduce the supply of oxygen and nutrients to the scar. Active collagen synthesizing fibroblasts will also be removed as the scar matures. Collagenase activity will increase and hence reduce the fibrous mass within the scar and decrease the scar volume (Velnar et al, 2009).

There are *in vitro* studies suggesting that keloid-derived fibroblasts showed overproduction of pro-inflammatory growth factors, particularly TGF beta (Robles et al, 2007; Seifert and Mrowietz, 2009; Shih et al, 2009). This group of growth factors plays an important role in inducing collagen production and fibroblasts proliferation. The over-expression of the receptor of TGF beta has also been observed in keloid-derived fibroblasts. Other studies report that fibroblasts isolated from keloid scars are more sensitive towards TGF beta activation when compared with normal fibroblasts

(Shih et al, 2009; Jagadeesan and Bayat, 2006). However, although the role of TGF beta in keloid formation sounds promising, it is considered to be a result instead of the cause for the wound healing defect (Campaner et al, 2006).

Other studies suggest that the formation of keloid scars is related to the abnormal balance between apoptosis and proliferation of fibroblasts (Lu et al, 2007; Luo et al, 2001; Ladin et al, 1998). At the end of the wound healing cascade, excess activated fibroblasts in granulation tissue are eliminated by apoptosis (Greenhalgh, 1998; Chodon et al, 2000). When the population of fibroblasts in granulation tissue is eliminated, it would develop into relatively acellular mature scar tissue (Velnar et al, 2009). It is often hypothesized that keloid scar derived fibroblasts exhibit reduced apoptotic behavior, and hence lead to an imbalance between collagen production and degradation, ultimately resulting in excessive proliferation of the scar. Reduced apoptosis of keloid-derived fibroblasts has been observed in many *in vitro* studies (Lu et al, 2007; Luo et al, 2001; Ladin et al, 1998; Chodon et al, 2000). However, the exact mechanism of how keloid scar fibroblasts develop such resistance to apoptosis is yet to be elucidated.

1.2 The Impact of Keloid Scarring

Keloid scars are seldom life threatening; however, the scar can grow large enough to cause disability, affecting mobility and joint movements. As a result of piercing and tattooing, keloids commonly develop in visible anatomical sites such as the earlobes and the upper part of the body, causing significant aesthetic deformity. Thus, although the disease itself is not life threatening, it can greatly affect the quality of life of the patients. In 2005, Bock et al recruited 100 outpatients with keloid and hypertrophic scars and demonstrated that the presence of the scar caused a greater psychological than physical effect on the patients. Patients commonly suffered from strong feelings of rejection and low self-esteem, especially in the younger age groups with decorative piercing. Also, the lack of satisfactory and reliable treatment contributes to the general negative feelings experienced by patients.

1.3 The Treatment of Keloid Scarring

There are a wide range of therapeutic interventions that have been tried in the treatment of keloids. The more commonly used modalities being 1) the application of silicon gel sheets, 2) intralesional steroid injection, 3) cryotherapy, 4) laser therapy and 5) surgical excision followed by radiation therapy. Numerous studies have been published to support the efficacy of the various treatment modalities; however, very few were shown

to be clinically significant. These studies typically employed neither placebo control nor blinding of the participants, with a short follow up period and small numbers. Moreover, it is difficult to quantify the improvements of the scars and little evidence-based techniques have been used (Robles et al, 2007). In 2006, Leventhal et al analyzed 39 studies that covered 27 different treatments for keloid. The group concluded that the effect of the treatments on improving keloid was not statistically significant. Therefore, it is still believed that none of the current treatments can be proved to have a predictably significant benefit on the clinical condition.

1.3.1 Silicon Gel Sheeting

While surgical excision of keloids is a straightforward modality that can provide immediate cosmetic correction, the recurrence rate is typically above 80 percent (Mustoe et al, 2002) and often leads to the potential for a larger keloid (Poochareon & Berman, 2003). Therefore, surgical excision is seldom used alone for treating keloid. The application of silicone gel dressings after surgical excision is one of the most common treatments adjuvant therapies (Juckett et al, 2009; Seifert et al, 2009). It is non-invasive and relatively inexpensive with positive evidence for its efficacy. In 2002 an international advisory panel on scar management recommended silicone gel sheet

therapy following surgical closure for the prevention of keloid and hypertrophic scar development (Mustoe et al, 2002). It is particularly useful for children who have a low tolerance for pain. In 2001, de Oliveira et al studied the effect on silicone gel sheets on scars and concluded that the scars appeared softer with reduced erythema, pain and itching. However, silicone gel sheeting was generally more effective on Hypertrophic rather than Keloid scars (Borgognoni, 2002).

1.3.2 Intralesional Steroid Injection

Intralesional steroid injection is considered as one of the first-line therapies for keloids. The recurrence rate is reported as 9 to 50 % (Juckett et al, 2009; Seifert et al, 2009; Mustoe et al, 2002). The treatment results of steroid injection can be improved when combined with surgery or cryotherapy (Robles et al, 2007; Seifert et al, 2009; Berman & Flores, 1997). Triamcinolone acetonide is typically used, and it is clinically shown to be able to help soften the scar and reduce pruritus and tenderness (Robles et al, 2007).

However, the pain associated with the intralesional steroid injection is one of the major drawbacks (Kelly, 2004; Musto et al, 2005). Also, up to 63 % of patients experienced side effects such as skin atrophy, hypo- or hyper-pigmentation, and the development of

telangiectasias (Robles et al, 2007). Therefore, intralesional steroid injection is not recommended for large keloid scars that require multiple injections.

1.3.3 Cryotherapy

Cryotherapy can be used alone or just before intralesional steroid injection to enhance the effects of the injection by inducing edema (Seifert et al, 2009; Lahiri et al, 2001). There are reports showing that cryotherapy was able to induce keloidal fibroblast differentiation towards a more normal phenotype and hence reducing collagen synthesis (Robles et al, 2007; Dalkowski et al, 2003). However, permanent hypo-pigmentation or hyper-pigmentation and skin atrophy is the common side effect (Kelly, 2009). Therefore, the therapy is not recommended for the darker skinned nations (Mustoe et al, 2002). Moreover, there is considerable pain and prolonged healing following the treatment (Kelly, 2004). Cryotheraphy is hence limited to the treatment of very small scars such as post acne scarring.

1.3.4 Radiotherapy

Radiotherapy can be used alone or in combination with the surgical removal of scars. Due to the concern that the radiation may induce carcinogenesis, this is usually considered as the last resorts for treating keloid when the scar is not responsive to other therapies. In one retrospective study (Ragoowansi et al, 2003), radiotherapy at 24 hours after surgical removal of keloid scars gave 80 % success rate with no recurrence of keloid at 5 years follow-up. Ogawa et al in 2003 also reported a 77 % success rate after 18 months follow-up.

However, besides the side effects of radiation therapy: erythema and hyper-pigmentation, the risk of carcinogenesis still remains the major concern for such therapy. Therefore, radiotherapy is not recommended for children and young adults (Botwood et al, 1999). It should be used for very severe keloids that are resistant to other therapies only (Ragoowansi et al, 2003).

1.3.5 Laser Treatment

The use of laser is a relatively new modality used in treating Keloid. The most promising results so far have come from the use of the Nd: YAG lasers (neodymium: yttrium-aluminum-garnet laser) and pulsed dye lasers (Mustoe et al 2002; Juckett et al

2009; Seifert et al, 2009; Tanzi & Alster, 2004). These lasers produce specific wavelengths of electromagnetic energy and are believed to be able to selectively destroy targeted structures in the skin. The use of the pulsed dye laser in conjunction with intralesional steroid injection has been reported to help to soften the scar and enhance the effects of steroid (Connell & Harland, 2000). In 2000, Kumar et al recruited 17 patients with keloids for Nd:YAG laser treatment. The group reported that nearly 60 % of the patients showed flattening of the scar with no recurrence of keloid for up to 5 years follow-up. Laser therapy was considered as an emerging treatment for keloid with potentially promising results (Mustoe et al, 2002). Its efficacy is yet to be confirmed by longer term follow-up and larger scale studies.

1.4 Summary

To conclude, little consistent success has been observed in the wide range of current therapies applied to keloid scarring, and each method has both drawbacks and benefits. Laser treatment for keloid scars, particularly those utilizing pulsed dye lasers and Nd:YAG lasers has gained popularity as emerging modalities for the management of keloid. Benefits of the method include being non-invasive, with little side effects. Laser of various frequency and intensity can possibly exert different effects on keloid, and

hence making it a potentially flexible treatment for keloids with different sizes and severity. Clinical studies using laser have already showed promising results on improvement of keloid scars. However, the mechanisms of the biological effects on tissues still remain unknown. In this thesis, the biological effects of 532 nm Nd:YAG laser using different energy levels on keloid-derived fibroblasts are investigated as to explore the possible mechanism of action of laser irradiation.

Furthermore, in the literature, a poor understanding on the pathology of keloids also makes the development of reliable therapies difficult. Without the support of sufficient biological research, treatments are typically developed by experience from clinical practices and this may be one of the reasons why existing treatments can seldom have consistent success with different keloid scars. It is very important to understand the fundamental pathogenesis in order to develop a more promising treatment for keloid. Ideally, cells, molecules or other external factors that directly lead to the formation of keloid scars have to be identified and targeted to yield effective treatment results. Given the observation that keloid-derived fibroblasts exhibit resistance to apoptosis during the course of scar maturation, the mechanism of how these cells develop such resistance may be the key in understanding the pathology of keloid. In order to gain an insight into the pathological apoptotic regulations of keloids scars, the apoptosis-related

gene expression profiles of keloid and normal fibroblasts are compared using microarray. The results can possibly help our understandings of the aetiology of the mysterious keloid scarring.

1.5 Aim of the study

The aim of this study is two fold. First of all, the pathological apoptosis regulation of keloid fibroblasts is investigated. The expression profiles of apoptosis-related genes of keloid scar and normal skin derived fibroblasts are compared and studied by using microarrays.

Secondly, the effect of Nd:YAG 532 nm laser on keloid-derived fibroblasts is studied. Nd:YAG 532 nm laser is an emerging modality to treat keloid scarring. In this study, the biological effects of Nd:YAG 532 nm laser radiation on keloid-derived fibroblast, including proliferation and apoptosis, are investigated.

2 Apoptosis and Keloid Pathology

2.1 Introduction

2.1.1 Apoptosis – an overview

Apoptosis or programmed cell death plays a critical role in nature. It is required during embryonic development, normal cell turnover and wound healing. It also plays important roles in the regulation of the immune system in response to physiological stimuli. It is also a process by which the cell 'commit suicide' in response to cell stress, cell damage or conflicting cell division signals (Evan & Littlewood, 1998; Nagata, 1997).

The maintenance of tissue function depends on the balance of the cell number which is controlled by apoptosis. Too much or not enough apoptosis can hence be very harmful. For example, cancers cells are difficult to be eliminated because they fail to respond to apoptotic signals. On the other hand, excessive apoptotic activity in neurons can lead to neurodegenerative disorders such as Parkinson's diseases (Thompson, 1995; Haass, 1999). Autoimmune disorders and central immune system phenomena are also related to the malfunction of the apoptotic control, which leads to failure in the elimination of

self-reactive lymphocytes and active immune cells after successful immune responses (Ashkenazi & Dixit, 1998).

Apoptosis is a single cell event that will not affect the neighboring cells. It is a cell death event characterized by a specific degradation of the cell's DNA and the loss of specialized membrane structures without triggering a significant inflammatory response (Rai et al, 2005). Central to apoptotic cell death is a family of proteases called caspases (cysteine-containing aspartate-specific proteases). A more complete understanding of the important role of these proteases play in apoptosis only began to emerge in the late 20th century (Adams & Cory, 1998; Ashkenazi & Dixit, 1998). Under normal circumstances, caspases are present as inactive proteins. These proteins are very powerful when activated and can cause massive destruction of key target proteins important to the survival of a cell. Therefore, there must be sufficient control over the activation of these proteins before a cell is irreversibly committed to apoptotic death. It is regulated under a number of pathways involving a huge number of molecules.

Apoptosis signals are regulated and transmitted either via the apoptotic receptors on the cell membrane (the extrinsic pathway) in response to external stress, or initiated from within the cell (the intrinsic pathway) following an order to "commit suicide" (Rai et al,

2005;Ashkenazi & Dixit, 1998; Andera, 2009). Through interactions of the molecules via different pathways, the "initiator caspases" (caspase 8 and 9) can be activated. The activated "initiator caspases" will in turn activate the "executioner caspases" (caspase 3,6 and 7) to execute apoptosis. Once the executioner caspases are activated, they will seek out and cleave their respective protein targets, hence dismantling the cell (Andera 2009; Lavrik et al, 2009; Nagata, 1997).

2.1.2 The role of apoptosis in wound healing

The role of apoptosis in wound healing was discussed in a review article written by Rai et al in 2005. Immediately following injury, surveillance neutrophils that reside in the tissues will quickly arrive at the injured sites. Cytokines are released to induce the proliferation and maturation of other inflammatory cells and start the wound healing cascade. Necrotic cells are removed and our immune system is activated to defend the body against invading microorganisms. When the wound healing response is initiated, neutrophils will be eliminated through apoptosis and consumed by macrophages to control and prevent a further inflammatory response. And finally, macrophages will also be eliminated by apoptosis when inflammation is down-regulated and a scar is formed as the end result of the wound healing cascade (Antoniades et al, 1994; Tidball et al, 1996).

Apoptosis is not only important in the regulation and control of the inflammatory cells, during the wound maturation phase, apoptosis is also crucial in determining the quality of the scar formation. The active granulation tissue consists of mainly active fibroblasts and collagen supplied by small blood vessels created during the active phase of the wound healing. When the scar matures, populations of the activated cells have to be eliminated by apoptosis. The active fibroblasts that can produce collagen in the scar tissue will be suppressed and eliminated by the apoptosis signal so as to prevent excessive fibrous scar tissue formation (Green, 1997; Rai et al, 2005). In addition, apoptosis signals are also important in collagen degradation by inducing collagenase activity and reducing the fibrous mass within the scar (Bian & Sun, 1997).

2.1.3 Apoptosis Research on Keloids

There are studies examining the differences in apoptosis regulation of keloids when compared with normal scar or normal skin. These differences are reported to lead to the failure in elimination of active fibroblasts when the scar matures and results in the pathologic scarring.

Ladin et al (1998) obtained biopsies from the center and the edge of keloid scars due to the observation that the scar is more active at the edge then the centre. The group used the TUNEL technique to stain the tissue and found that the number of apoptotic cells was lower when compared to normal skin. They also observed that the number of apoptotic cells decreased near the edge of the lesions. The group also performed experiments on monolayer fibroblast cultures and found that there was reduced apoptosis in keloid fibroblast cultures when compared with normal skin fibroblast cultures.

Luo et al (2001) also performed experiments on fibroblasts derived from both keloid and normal skin in a monolayer culture model. The keloid scars were horizontally divided into superficial, central, and basal groups and gave rise to three separate cell cultures. The number of cells that were undergoing apoptosis was identified using the TUNEL technique. Results showed that the number of apoptotic cells in keloid-derived fibroblast cultures of all the three regions was less than half when compared to that of normal fibroblasts. The group concluded that keloid-derived fibroblasts have reduced apoptosis when compared to normal fibroblasts derived from normal skin, which agreed with the results of Ladin et al in 1998.

Given the fact that keloid scars seldom develop in the palm and sole, Chipev et al (2000) chose palmer fibroblasts from people with no keloid history as a control, and compared those with keloid fibroblasts. In his study both cell cultures were starved from serum supplement and the apoptotic behaviors of the cells were observed using Annexin V and TUNEL assay. While normal fibroblasts underwent apoptosis due to serum starvation, keloid fibroblasts were resistant to the stress created. The group concluded that fibroblasts derived from keloids were more resistant to apoptosis when compared to normal skin.

Lu et al (2007) raised monolayer cell cultures of fibroblasts isolated from peripheral and central area of keloid scars. The group induced Fas-mediated fibroblasts apoptosis using anti-Fas antibody in monolayer fibroblast culture models, and found that both peripheral and central keloid-derived fibroblasts were resistant to Fas-mediated apoptosis. Such resistance was not observed in normal fibroblasts. Lu's results agreed with Chodon et al (2000) in a similar study where Fas-mediated apoptosis was induced in monolayer fibroblast cultures. The group also found that the expression level of Fas-receptor was the same in the keloid and the normal control groups, suggesting that there may exist some molecules in keloid that impair the Fas-mediated apoptosis pathway.

To summarize, studies that obtained punch biopsies of keloid scars revealed decreased apoptotic cells when compared with normal skin. The distribution of the apoptotic cells was decreased around the edge of the scars. Studies that used *in vitro* cell culture models suggested that keloid-derived fibroblasts are refractory to apoptosis when cell stress was introduced. However, the exact mechanism of how this resistance to apoptosis is developed is yet to be understood.

In order to further understand the pathogenesis of keloid scarring, the gene expression studies are required. The expression levels of a particular gene can be revealed using Northern blotting analysis. One of the most significant findings in the studies of gene expression was that keloid has a clear up-regulation of TGF beta gene that contributed to the excess production of collagen and the enhanced proliferation of fibroblasts (Chau et al, 2005; Messadi et al, 1998; Naitoh et al, 2001; Phan et al, 2003; Zhang et al, 2004). These results are very promising; however they are often considered to be a consequence of an abnormal wound healing process instead of the cause (Campaner et al, 2006). Apoptosis is another important area in the pathological studies in keloid. However, since the functions of many apoptosis-related proteins are not yet fully understood and are also heavily interrelated, studying the gene expression one by one is not effective in revealing the full picture of the apoptotic pathways. Recently, the comparison of keloid

and normal fibroblasts in their apoptotic behaviors has been made more effective by comparing the differences in the gene expression levels using microarrays.

2.1.4 Microarray analysis of Keloid Scars

Cell behavior is governed by protein production, which is in turn regulated by the transcription of mRNAs. In 1995, Schena et al were the first to extract mRNA from cells and quantify the relative expression levels using microarray. This publication has had a great impact in biological research not only because it has made gene expression studies more effective by comparing thousands of genes simultaneously. More importantly, it enables researchers to study the inter-relationship between different genes and suggest their unique association to the disease (Chin et al, 2006).

Keloid researches utilizing microarray began at the turn of the 20st Century. In 1999, Sayah et al recruited 4 keloid patients and another 4 normal volunteers with matched sex and age. 64 apoptosis-related genes were compared between the keloid group and the normal group, 8 apoptosis-related genes are identified to be differently expressed.

Chen et al (2003) compared 8400 genes in 3 patients with keloid scars, and the gene expression of each of them was normalized with the corresponding normal dermal fibroblast culture expression levels. The 8400 genes selected include collagen production genes, fibronectin and proteoglycan genes, growth factor and receptor genes, and apoptosis-related genes *etc*. 402 genes were shown to have different expression levels and that included 4 apoptosis-related genes: ASK1, KIAA0018, p21, and SARP1 (secreted apoptosis-related protein 1).

Satish et al (2006), on the other hand, compared 22284 genes in keloid scars derived from 3 patients, to 3 healthy individuals. The patients in the keloid group and the control group were randomly selected with different sex and age. The position where the skin samples were collected was also different. In their findings, 43 genes were over-expressed in keloid while 5 were under-expressed. Out of the 43 over-expressed genes, the ones that are related to apoptosis were ASY and PEA 15 (phosphoprotein enriched in astrocytes 15, shown to oppose Fas-induced apoptosis).

A summary of competent recent papers using microarray to compare keloid scar and normal control are shown as follows:

Table 1 Summary of recent papers on gene array analysis of keloid

authors	year	normal control (sample size)	keloid (sample size)	array type	number of genes analysized	Ap related genes
Satish *et al*	2006	random normal skin (n=3)	earlobe and lumber region (n=3)	cRNA array	22284	ASY PEA 15
Seifert *et al*	2007	normal skin from other individuals with matched age and sex (n=3)	spontaneous keloid chest abdoment upper chest (n=3)	cRNA array	whole human genome	ADAM12 CCAR1
Chen *et al*	2003	normal skin from the same patient (n=3)	burn (n=3)	cDNA array	8400	ASK1 KIAA0018 p21 SARP1
Sayah *et al*	1999	normal scar of individuals with matched sex and age (n=4)	chest, head and neck (n=4)	cDNA array	64 apoptosis related	DAD-1 c-myc transcription factor glutathione S-transferase glutathione S-transferase microsomal glutathione perosidase TRADD NIP3 HDLC1
Smith *et al*	2008	not mentioned (n=5)	not mentioned (n=5)	cRNA array	38500	none
Naitoh *et al*	2005	normal skin from the same patient (n=3)	not mentioned (n=3)	cDNA array	9182	none

All these studies reported that some apoptosis-related genes were differently expressed in keloids. However, little consistency was found. Also, the authors did not further discuss how these expression profiles may contribute to the pathology of keloid scars.

In order to reveal a clearer picture of the apoptotic regulations in keloid, in this study we used a cRNA gene array targeting on 112 apoptosis-related genes to investigate 3 pairs of keloid and normal skin samples obtained from the same patient. Genes that were differently expressed were identified, and the possible roles they play in keloid scarring are discussed.

2.2 Material and methods

2.2.1 Cell Culture

All procedures were approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. Fibroblast cultures were obtained by explants techniques using keloid scar or normal skin tissue specimens discarded during surgical procedures. Normal skin and keloid scar specimens were collected from 3 patients

Sample	Gender	Age
ZYZ	F	12
LWS	F	6
101	F	46

Skin specimens were collected in sterile grass bottles with Dulbecco's modified eagle medium (Gibco-BRL, Life Tech, CA, USA) containing anti-biotics and anti-mycotics solution: 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone (Gibco-BRL, Life Tech, CA, USA). Dispase solution was prepared by dissolving 5 mg of dispase powder (Roche applied science, Germany) in 1 ml DMEM. The tissue sample was rinsed three times with phosphate buffered saline (PBS) and was immersed in the dispase solution overnight at 4°C. The dermis was separated from the epidermis by forceps and was then minced by scalpel and scissors into 1 - 2 mm cubes and evenly distributed on 100 mm tissue culture plates (Nunc, Rochester, NY). The tissue was incubated for 2 hours at 37 °C, 5 % CO_2, to facilitate explants attachment followed by 2 ml FBS incubation overnight. The following day, 2 ml of DMEM containing 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone was added and the final concentration of FBS in the medium became 50 %. Medium was not changed during the first 4 days and afterwards, DMEM + 10 % FBS was used. Medium was changed twice a week until outgrowth of fibroblasts is observed. Fibroblasts were

subcultured by trypsinization using 0.25 % trypsin- EDTA (Gibco BRL) once 90 % confluence was reached and expanded in 1:5 ratio in DMEM + 10 % FBS. All fibroblasts used in this study were from passage 3 - 7.

2.2.2 Microarray Analysis

Isolation of RNA

RNA was isolated using ArrayGrade™ Total RNA Isolation Kit (SuperArray Bioscience Corporation) according to the manufacturer's protocol. The filter columns, collection tubes, buffers and reagents were provided by the kit.

5 x 10^6 cells were harvested from a 100 mm culture dish. Cells were first pelleted in a 1.5 ml RNase-free tube and lysed using 350 µl of Lysis and Binding buffer. The filtrate containing RNA was collected after 11000 g centrifugation for 1 min through a filter column. 350 µl of 70 % ethanol was added to the filtrate and was then desalted by adding 350 µl of Desalting Buffer followed by 1 min of 11000 g centrifugation through a clean spin column. The spin column was washed 3 times using washing buffers provided by the kit followed by 30 s centrifugation at 8000 g. After the last wash, the tube was centrifuged for 3 min at 11000 g to dry the column completely. 50 µl

RNase-free water was added to the spin column to dissolve the purified RNA, and it was collected in a RNase-free 1.5 ml microcentrifuge tube after centrifugation for 1 min at 11000 g.

The total RNA extracted was quantified by measuring the absorbance at 230, 260 and 280 nm. 49 µl TE (pH 8.0) was dispensed into UV transparent microcuvette and 1 µl of RNA sample was added. 1.5 µg of total RNA of each sample was also characterized on a denaturing 2.0 % agarose gel containing ethidium bromide (0.5 µg/ml). The total RNA sample that contained sharp 28S and 18S rRNA bands and peaks without smearing was stored at -80 °C.

cRNA Target Labeling for Oligo Hybridization

For each RNA sample, 3.0 µg of total RNA, 1.0 µl of TrueLabeling primer (Oligo GEArray Reagent Kit) together with RNase-Free H_2O were added to a final volume of 10 µl followed by incubation at 70 °C for 10 min. Afterwards, 4 µl RNase-free H2O, 4 µl of 5X cDNA Synthesis Buffer, 1 µl RNase Inhibitor and 1 µl cDNA Synthesis Enzyme Mix was added to each sample and was incubated at 42 °C for 50 minutes followed by 75 °C for 5 minutes then cooled to 37 °C. 16 µl of 2.5X RNA Polymerase

Buffer, 2 µl Biotinylated-UTP and 2 µl 10 mM RNA Polymerase Enzyme were mixed to a final volume of 20 µl at room temperature and was added to each sample. The tube was incubated for overnight at 37 °C to yield cRNA.

cRNA purification was then performed using SuperArray ArrayGrade™ cRNA Cleanup Kit. 50 µl of RNase-free H2O, 315 µl Lysis & Binding Buffer and 315 µl ACS-Grade 100 % ethanol was added and centrifuged for 30 s at 8000 g through a spin column. The cRNA as filtration residue was washed twice using washing buffer followed by centrifugation for 30 s at 8000 g. After the last wash, the tube was centrifuged for 3 min at 11000 g to dry the column completely. 50 µl RNase-free water was added to the spin column to dissolve the purified cRNA, and it is collected in a RNase-free 1.5 ml microcentrifuge tube after centrifugation for 1 min at 11000 g.

Oligo GEArray® microarray

A pre-wetted array membrane inside a hybridization tube with 2 ml of pre-warmed GEAhyb Hybridization solution was incubated inside the hybridization oven at 60 °C for 2 hours with continuous agitation at 5 rpm. Afterwards, 3 µg of biotin-labeled cRNA in

0.75 ml pre-warmed GEAhyb Hybridization Solution was added for overnight incubation at 60 °C with agitation at 5rpm.

The following day, the array membrane was washed with 5 ml Wash Solution 1 followed by 5 ml Wash Solution 2 at 60 °C with agitation at 10 rpm for 15 minutes. 2 ml GEAblocking Solution Q was added to the hybridization tube and incubated for 40 min with agitation at 10 rpm in room temperature. Afterwards, 2 ml Dilute AP-SA Buffer was added to the tube and was incubated for 10 min with agitation at 10 rpm. The membrane was then washed with 4 ml 1x Buffer F for 5 min with agitation at 5 rpm for 4 times and was rinsed twice with 3 ml Buffer G. Finally, 1.0 ml CDP-Star chemiluminescent substrate was added to the hybridization tube and was incubated for 3 min at agitation 10 rpm. The membrane was then placed into a plastic sheet protector. Image was acquired by exposing the membrane on a film for 1 – 5 minutes depending on the intensity of the signal.

2.2.3 Real-time PCR

cDNA Primers for real-time PCR were designed using Light-Cycler Primer Design software, and the respective sequences were as follows:.

TNFRSRF12A

Human TNFRSF12A forward: CTGGCTCCAGAACAGAAAGG

Human TNFRSF12A reverse: GGGCCTAGTGTCAAGTCTGC

TNFRSRF6B

Human TNFRSF6B forward: TCAATGTGCCAGGCTCTTC

Human TNFRSF6B reverse: GCCTCTTGATGGAGATGTCC

TNFRSF1B

Human TNFRSF1B forward: GTCGGTAAGTTGAATGG

Human TNFRSF1B reverse: TCATGTCCTCATGTGG

TNFRSF10D

Human TNFRSF10D forward: AGTACTTGACCCATGC

Human TNFRSF10D reverse: GCCTAAAACGACCCTT

TNFRSF11B

Human TNFRSF11B forward: CCTAACTGGCTTAGTG

Human TNFRSF11B reverse: GTGAGGTTAGCATGTC

According to the manufacturer's protocol, a master mix for the LightCycler PCR reaction was prepared containing 5 µl PCR grade water, 1 µl forward primer (0.5 µM), and 1 µl reverse primer (0.5 µM), 2 µl LightCycler Fast Start DNA SYBR Green I

Master Mix (Roche Diagnostics). The master mix was prepared in multiples of the volume described above sufficient for the total number of reactions to avoid pipetting error. 9 µl of the LightCycler master mix was pipetted into the Light-Cycler glass capillaries on a pre-cooled LightCycler Sample carousel, and 1µl sample cDNA was added as a PCR template.

Real time PCR Experimental runs were performed in duplicate under the following conditions: Denaturation – at 95 °C for 10 min

Amplification and quantification (45 Cycles) - Single fluorescence measurement at 95 °C for 10 s, then at annealing temperature of individual primers for 10 s

Annealing temperature:

TNFRSF6B- 56°C

TNFRSF11B- 55°C

TNFRSF12A- 56°C

TNFRSF1B- 56°C

TNFRSF10D- 57°C

Melting curve –fluorescence was continuously measured at 60 – 95 °C with a heating rate of 0.18 C/s.

Cooling – 40 °C

Results were analyzed using Light-Cycler Relative Quantification Software (Roche Diagnostics) and expressed as respective ratio with respect to GAPDH.

2.2.4 Statistical Test

The results were expressed in keloid/normal expression ratios. Under n=3, 2-sided Wilcoxon Signed Rank Test has been performed on the real-time PCR results to test if the expression ratios were significantly different from 1.

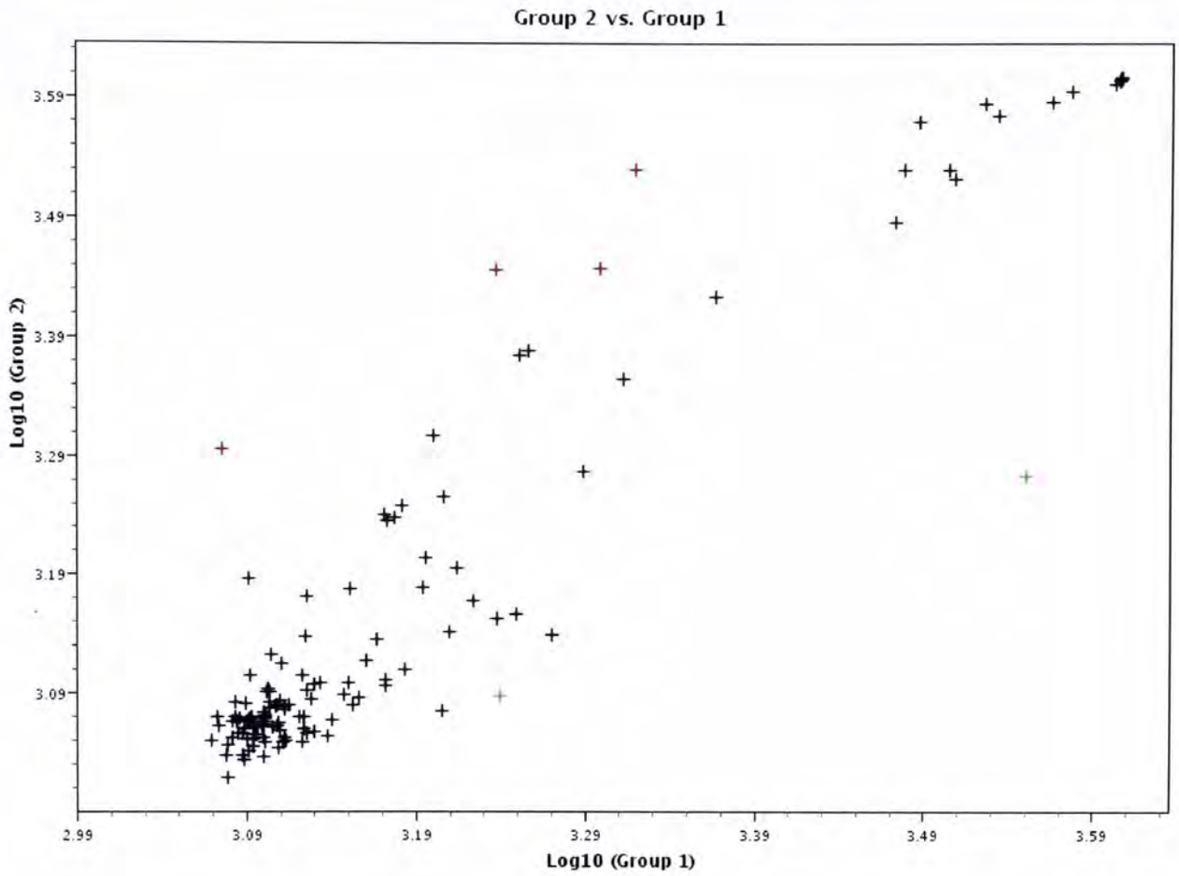
2.3 Results

2.3.1 Apoptotic gene profiles in keloid versus adjacent normal fibroblasts

Based on the data obtained from the gene array, the expression levels of each gene were quantified and plotted as scatter plots by the GEA suit program. The genes expressed in keloid scar derived fibroblasts were plotted in the Y-axis against their corresponding normal skin as control in the X-axis. The significant level was chosen to be a 1.4 fold difference in expression level to screen out a reasonable number of genes for further analysis. Apoptosis-related genes that are significantly different in expression levels have been identified and listed as below respectively for each pair of the three samples.

The first pair:

K1 (patient 101)



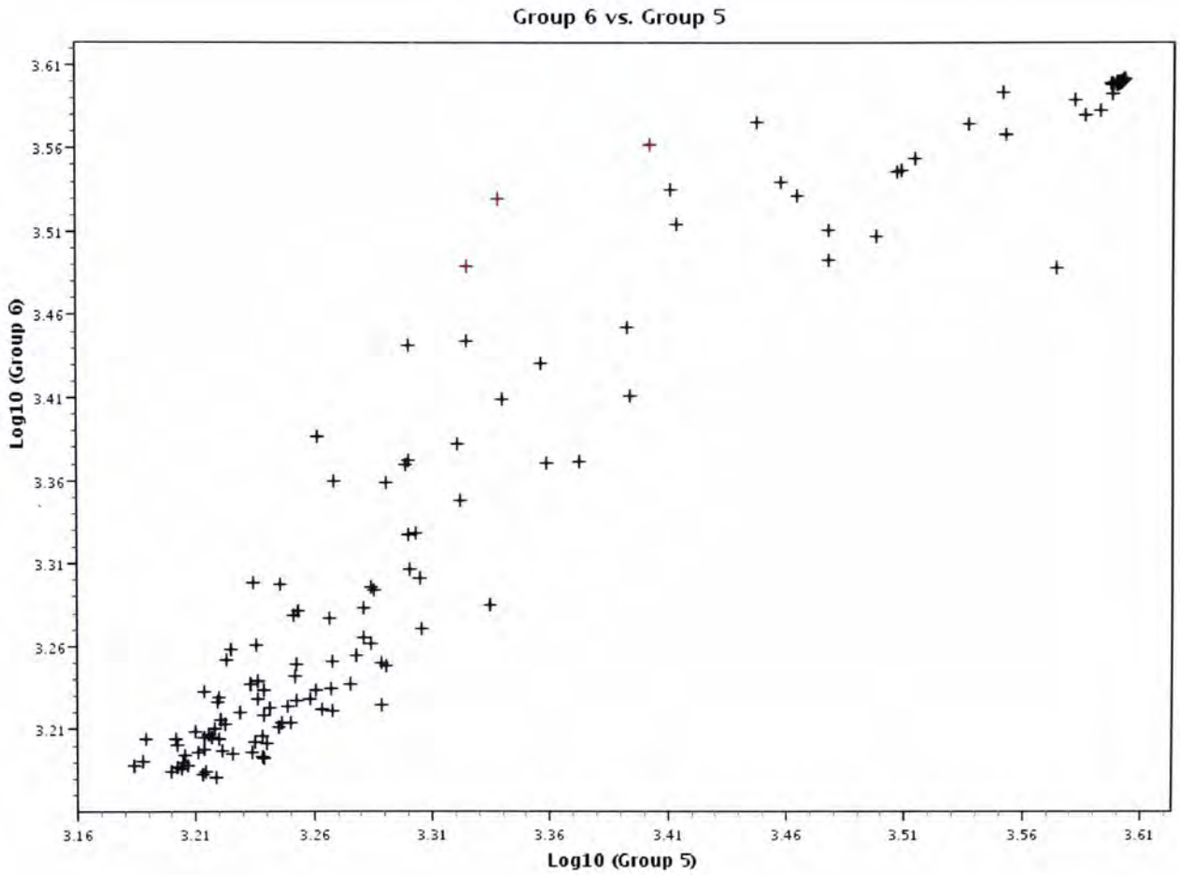
Genes up-regulated in keloid	fold difference
CASP4	1.63
DAPK2	1.62
FASLG	1.66
TRADD	1.41

Genes down-regulated in keloid	fold difference
TNFRSF11B	0.53
TNFRSF1B	0.70

Figure 1. First pair of keloid scar/ normal skin gene expression analysis

The Second pair:

K2- (patient LWS)

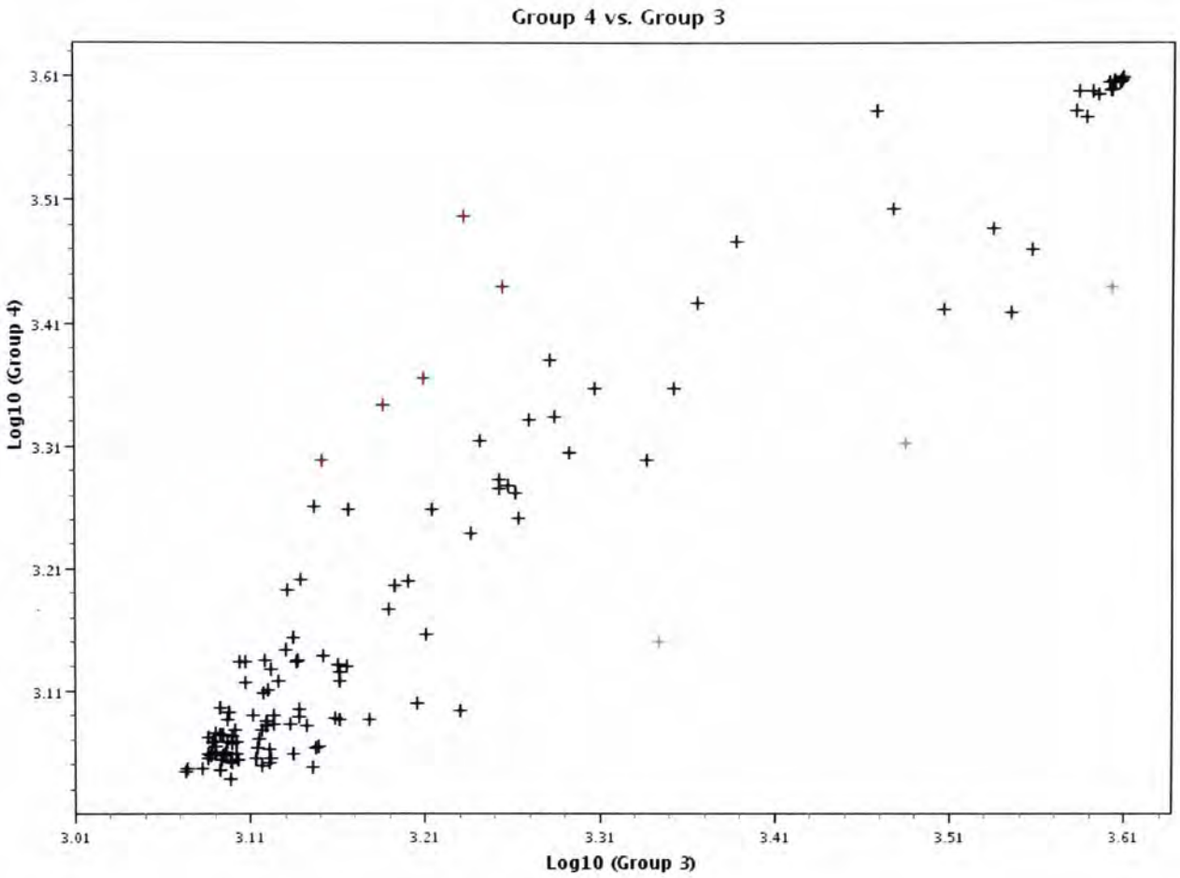


Genes up-regulated in keloid	**fold difference**
TNFRSF12A	1.46
TNFRSF6B	1.56
TP53	1.45

Figure 2. Second pair of keloid scar/ normal skin gene expression analysis

The third pair:

K3- (patient ZYZ)



Genes up-regulated in keloid	**fold difference**
BAX	1.43
CARD8	1.40
TNFRSF10D	1.44
TNFRSF12A	1.53
TNFRSF6B	1.84

Genes down-regulated in keloid	**fold difference**
BNIP3L	0.67
TNFRSF11B	0.69
TNFRSF1B	0.64

Figure 3. Third pair of keloid scar/ normal skin gene expression analysis

Out of the three pairs of samples, the genes that showed significant difference in expression levels in at least 2 pairs of samples have been identified and listed as below.

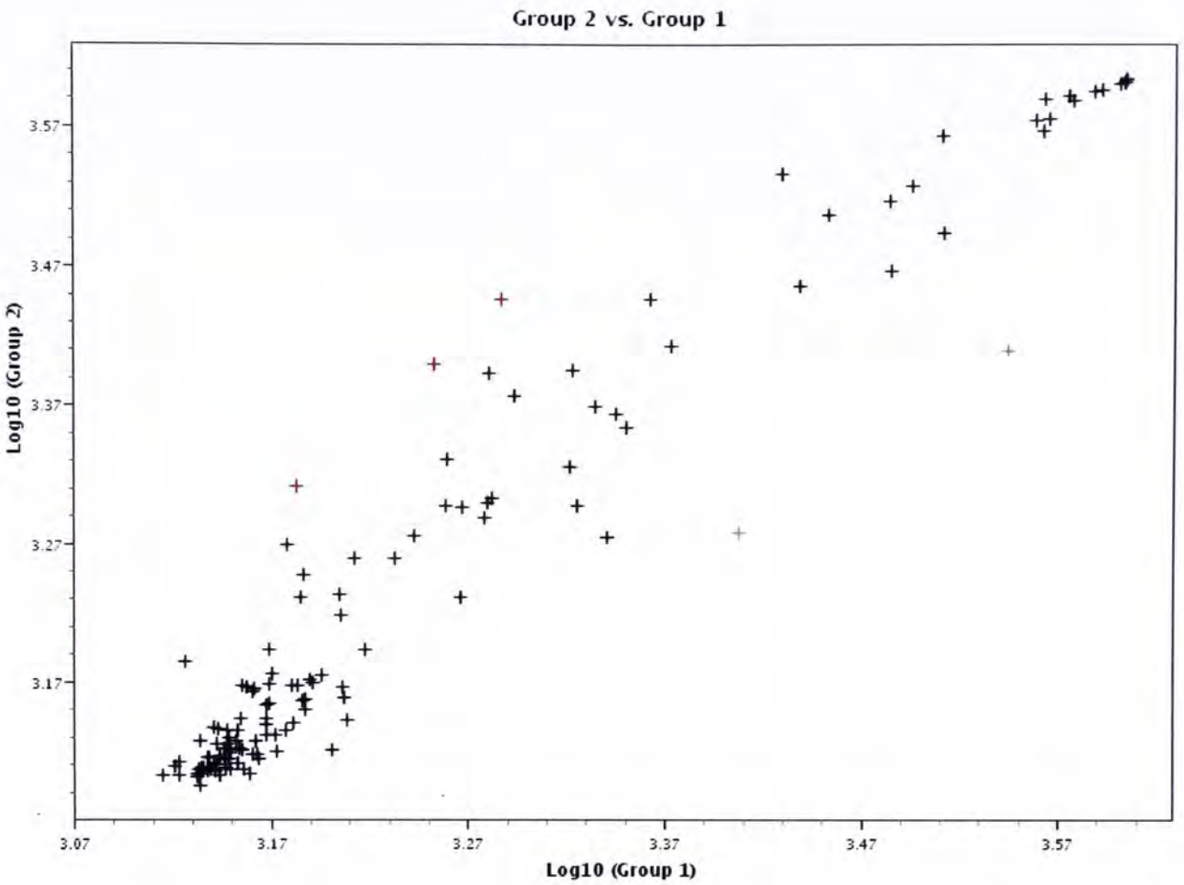
TNFRSF6B

TNFRSF12A

TNFRSF11B

TNFRSF1B

To further confirm the consistency in the difference in the gene expression levels, the averaged gene expression levels in keloid-derived fibroblasts were obtained by grouping all keloid samples and taking the mean. They were compared to the average gene expression levels of normal skin samples. The significant level was chosen to be a 1.3 fold difference in expression levels to screen out a reasonable number of genes for further analysis. The comparison results are shown below.



Genes up-regulated in keloid	fold difference
TNFRSF12A	1.40
TNFRSF6B	1.45
TNFRSF10D	1.35

Genes down-regulated in keloid	fold difference
TNFRSF11B	0.73
TNFRSF1B	0.74

Figure 4. Analysis on the consistency in the difference in gene expression levels

2.3.2 Real time PCR confirmation

The 4 genes that were obtained using the pairwise comparisons were also found in this averaged analysis. This suggested that the difference in expression levels of these genes was significant and consistent. Real time PCR has been performed to confirm and quantify the difference in expression levels. The results were as follows:

Fold difference in gene expression

	TNFRSF10D	TNFRSF11B	TNFRSF1B	TNFRSF6B	TNFRSF12A
K1/N1	2.00	0.31	0.36	1.86	1.54
K2/N2	1.37	0.16	0.57	1.79	1.83
K3/N3	2.76	0.88	0.84	3.59	4.89
Average fold difference	**2.04**	**0.45**	**0.59**	**2.41**	**2.75**
SD	**0.69**	**0.38**	**0.24**	**1.02**	**1.86**

n=3

p=0.109 for all groups (2-sided Wilicoxon Signed Ranks Test)

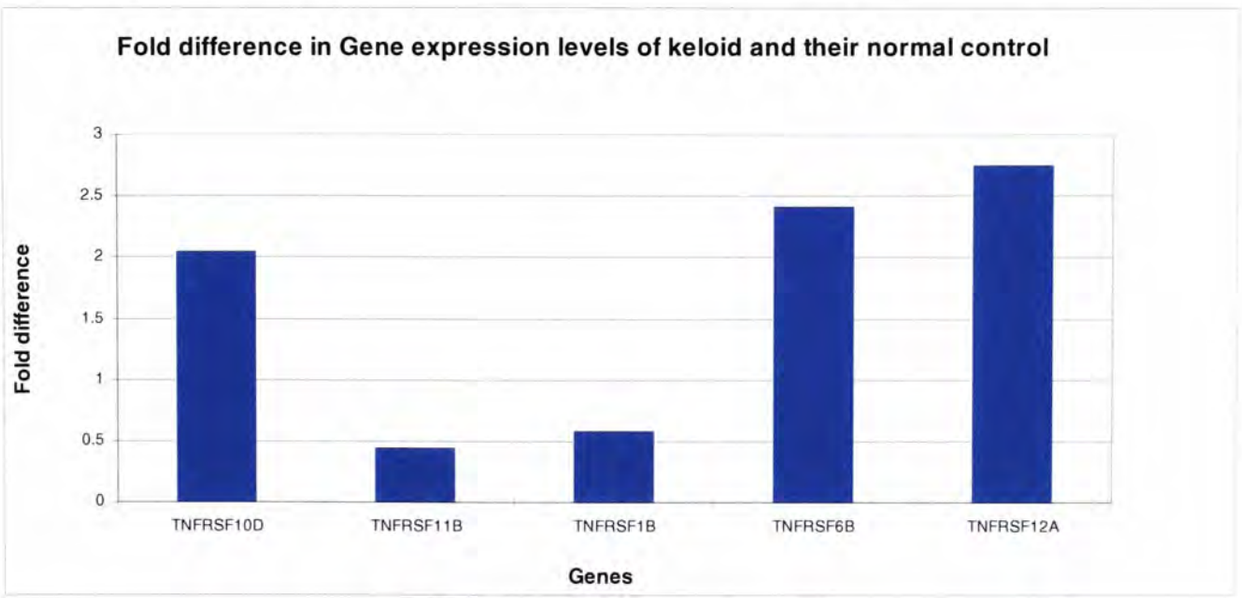


Figure 5. Results of Real Time PCR analysis on gene expression

2.4 Discussions

2.4.1 Gene Array Experimental Design Discussions

The choice of normal control in gene array experiments was particularly important as it was acting as a threshold (baseline) for gene expression levels. Deviations from the threshold suggested there are up or down regulations of gene expression. Therefore, the genes identified in the microarray analysis were dependent on the choice of control group. Existing studies have been using various normal controls including:-

1) Adjacent normal skin from the same keloid patient (Chen et al, 2003)

2) Random normal skin from volunteers without keloid history with unmatched sex, age and site (Satish et al, 2006)

3) Normal skin from volunteers without keloid history with matched sex, age and site (Sayah et al, 1999; Seifert et al, 2005)

Despite the importance of the normal control, many published papers failed to discuss the choice of their control. Some even failed to disclose the source of the normal sample, adversely affected the impact of the published results.

Studies that have chosen random normal skin sample from volunteers with no keloid history as control have an obvious advantage that the sampling bias could be removed. Provided that the normal pool is large enough and the volunteers are well chosen to be truly non-keloid-prone, the net effect of the individual variations can become very small. The group will then represent a characteristic normal threshold of gene expression levels, and reveal the deviations in keloid lesions.

However, given the relatively small example size in general for keloid studies, it is questionable that whether the published papers could ever reach a scale that is large enough to remove the individual variations, which is essential for to the following reasons:-

1) The structure of skin changes drastically across ages. Therefore, the skin samples obtained from individuals with different age groups might not be comparable.

2) The metabolic rate for the younger individuals is far higher than that of the elderly. Apoptosis-related gene expression may also be drastically different. This potentially huge difference in apoptosis-related gene expression levels can obscure the significance of expression level differences between the normal and the keloid.

3) Skin quality such as thickness, color and strength varies in different parts of body. Therefore, skin samples obtained from different part of body with different quality might

not be comparable.

Because of this, normal samples from individuals with matched age and sex may be a better alternative. However, in addition to the difficulties in collecting the samples given the tightened criteria, the normal pool neither represents a random normal control nor does it eliminate (though minimized) individual variations.

For small sample size keloid scar studies like ours, due to research constraints, using the patient's own normal skin as control would be the most effective, as individual variations are completely eliminated. Comparison results could clearly reveal the deviations of keloid-derived fibroblasts from normal. Even small yet consistent difference, which could be masked under comparisons with random normal controls, could be identified. However, since there has been reported family linkage for keloid formation, such research design may raise concerns on whether the normal skin of keloid prone individuals is biased.

2.4.2 Gene Array Results Discussions

2.4.2.1 The Death Receptors and Death Ligands

Interestingly, all the genes identified in this study are apoptosis death receptors. Death receptors are involved in the extrinsic apoptosis pathway and will interact with the death ligands to mediate apoptosis (Nagata, 1997; Andera, 2009). Death receptors mainly exist as a type I transmembrane protein, with an extracellular region interacting with the death ligands, and an intracellular region known as the death domain (Guicciardi and Gores, 2009). When death ligands interact with their respective death receptor extracellularly, a cascade of intracellular events will be triggered through their death domain and will eventually lead to apoptosis. The Fas ligand (FasL, also known as CD95L), tumor necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL) are subsets of death ligands and shared comparable protein structures (Andera, 2009; Mahalingam, 2009; Zhang et al, 2000; Guicciardi and Gores, 2009).

The death receptors such as Fas (receptor for FasL, also known as CD95 or TNFRSF6B), TRAIL receptor and TNF receptors are expressed in a wide range of normal cells (Gupta et al, 2001). However, some death ligands are secreted mainly by white blood cells only. For example, TNF is mainly secreted by activated T cells and macrophages while FasL

is mainly secreted by cytotoxic T cells and natural killer cells (Gupta et al, 2001; Kumar & McNerney, 2005; Strasser et al, 2009). This is actually how white blood cells get rid of tumor cells, virus infected cells and badly damaged cells, by secreting death ligands and inducing apoptosis (Strasser et al, 2009). However, the case is a little bit different for TRAIL. TRAIL is expressed in a wide range of normal cells which also have the TRAIL-receptors on their cell membrane (Zhang et al, 2000; Mahalingam et al, 2009). Therefore, to avoid induction of apoptosis that is not necessary, there must be a system to protect the cells.

The tumor necrosis factor receptor superfamily (TNFR superfamily) consists of two main subgroups of receptors: the death receptors and the decoy receptors (Andera, 2009; Guicciardi and Gores, 2009). They are homolog proteins that are very similar in structure but serve very different functions. The death receptors generally have a death domain in the cytoplasmic regions. When activated, they are able to trigger a series of cytoplasmic events that would lead to apoptosis (Nagata, 1997); decoy receptors generally do not have an effective cytoplasmic region and will not be able to directly trigger apoptosis cascades (Ashkenazi & Dixit, 1999). Instead, they protect the cells from apoptosis by competing with the death receptors for death ligands. Over-expression of decoy receptors can potentially inhibit apoptosis under their respective pathway.

Death decoy receptor 1 and 2 (DcR1 and DcR2) are decoy receptors that have very similar structure when compared with TRAIL-R1 and TRAIL-R2 respectively except for their cytoplasmic region (DcR1 does not have a cytoplasmic region while DcR2 only has a truncated cytoplasmic tail) (Zhang et al, 2000; Mahalingam et al, 2009; Guicciardi and Gores, 2009). Note that the decoy receptor DcR2 is also known as TNFRSF10D. The Fas decoy receptor is DcR3, also known as TNFRSF6B. Both of them are up-regulated in our study.

2.4.3 Down-regulations of TNFRSF1B in Keloid-derived Fibroblasts

TNFRSF1B, more commonly known as TNFR2, is one of the two main Tumor Necrosis Factor receptors (TNF receptors). TNF plays important roles in activating and modulating body's inflammatory response. It is highly pro-inflammatory and its expression could be found in a wide range of cells in our immune system such as monocytes, macrophages, mast cells, T and B lymphocytes *etc.* (Bradley, 2008)

TNFR1 and TNFR2 are by far the most well known receptors for TNF. They are most commonly found on the surface of circulating T cells (Bradley, 2008). Besides TNF, TNFR1 and TNFR2 can react with the same or completely unrelated molecules, adding

complexities into the role and the effects on interactions between the two receptors. TNFR1 is relatively well studied in terms of its activation pathways and function. Its activation will lead to pro-inflammatory responses and has been considered the main receptor for TNF, while TNFR2 was considered only acting an auxiliary function (Holtmann et al, 2002). However, studies have emerged to suggest that there are differentials in expressions of the two receptors observed in various types of cells under different pathological conditions (Al-Lamki et al, 2001; Al-Lamki et al, 2005; Hamid et al, 2009), hinting that there may be unknown roles of TNFR2 in the inflammatory response yet to be discovered. More recent studies even revealed that TNFR2 appeared to signal both complementary and opposing effects to TNFR1 (Bradley, 2008).

Al- Lamki et al (2005) studied the effect of both receptors in normal kidney cells and obtained that TNF-TNFR1 pathway could trigger tubular cell apoptosis while TNF-TNFR2 pathway promoted proliferation. Hamid et al in 2009 induced heart failure in wild type TNFR1 -/- and TNFR2 -/- mice via coronary ligation. By examining the cardiomyocytes, the group obtained that TNRF1 exerted pro-apoptotic effects to the cells while over-expression of TNFR2 attenuated the apoptotic signals.

While the role of TNFR2 in kidney and heart muscle cells seems to be opposing apoptosis and inflammation, Kafrouni et al in 2003 demonstrated the opposite effect in the liver. The group studied the antiviral-immune response on TNFR1 and TNFR2 deficient mice. Results showed that down-regulation of TNFR2 exhibited decreased levels of FasL expression to combat virus. The group concluded that the TNF-TNFR2 interaction was essential to anti-viral immune response in liver via the FasL pathway.

Existing studies so far are not capable of drawing a concrete conclusion on the role and action of TNF-TNFR2 interactions. However, it is clear that TNFR2's action is dynamic and heavily tissue specific. It may also depend on the availability of TNF and the relative abundance of TNFR1. In our studies we have identified a significant down-regulation of TNFR2 in keloid-derived fibroblasts when compared with normal. Whether or not the observation contributes to the apoptotic defects of keloid lesions still remains to be unfolded.

2.4.4 Up-regulations of TNFRSF10D in Keloid-derived Fibroblasts

TNFRSF10D is more commonly known as DcR2, a member of the TRAIL pathway. The TRAIL pathway is a very interesting apoptosis pathway. It was first discovered by

Wiley et al in 1995. Soon after its discovery, the unique feature of the apoptosis pathway was quickly revealed. TRAIL has been shown to be able to induce apoptosis in transformed cells, for example the cancer cells, but have minimal cytotoxic effects on normal cells (Mariani & Krammer, 1998; Walczak et al, 1999). It has drawn considerable attention throughout the years and has been studied as a potential therapeutic target for treating cancers (Mahalingam et al, 2009).

TRAIL is mainly expressed by activated T cells. It exerts its apoptotic function via interacting with the death receptors on cell surface, DR4 and DR5 (Chaudhari et al, 2006). Earlier studies often speculate the decoy receptors DcR1 and DcR2 may play major roles in protecting the normal cells from apoptosis (Wiley et al, 1995; Pitti et al, 1996). Cancer cells, on the other hand, may express less decoy receptors when compared with death receptors and hence become more susceptible to TRAIL-induced apoptosis (Mahalingam et al, 2009).

However, more recent studies revealed that the protection mechanism on normal cells was far more complicated than what had been expected. For example, Jo et al in 2000 demonstrated that TRAIL induced significant apoptosis in normal human hepatocytes. The group concluded that any cancer therapies targeted on TRAIL was not suitable for

treating liver cancer. Furthermore, Zhang et al in 2000 studied the effect of TRAIL on varies types of normal cells including fibroblasts from human lung, melanocytes, and human umbilical vein endothelial cells. The group demonstrated that all the three types of normal cells were resistant to TRAIL induced apoptosis. However, the mechanism for developing such resistance varied across the different types of normal cells. Human umbilical vein endothelial cells mainly combated TRAIL via the expression of the TRAIL decoy receptor DcR1. The group showed that removal of the decoy receptor could increase the cells susceptibility to TRAIL induced apoptosis. On the contrary, the group could only observe expression of TRAIL death receptor DR5 on normal human fibroblasts, while the expression of decoy receptors DcR1 and DcR2 were not detectable. While normal fibroblasts were shown to be resistant to TRAIL induced apoptosis, the protection did not seem to come from the decoy receptors alone. In their studies the group tried to determine if there is any other TRAIL inhibitors existed which contributes to the resistance. However, the molecules covered under the scope of the study were only minimally detectable.

While Jo et al (2000) demonstrated that normal fibroblasts isolated from lung only expressed the death receptor DR5, Miyashita et al in 2004 observed that fibroblasts isolated from rheumatoid arthritis, an auto-immune disease, expressed both DcR1 and

DcR2. Synovial fibroblasts isolated from rheumatoid arthritis are known to show resistance to apoptosis. However, the group did not compare the expression levels of the decoy receptors with the normal counterpart as control, nor did they determine whether the cells' resistance to apoptosis was related to the expression of the decoy receptors.

We are the first group to observe an up-regulation of TRAIL decoy receptor DcR2 up-regulation in keloid scar derived fibroblasts. From existing studies, we have determined that normal fibroblasts express more death receptors on TRAIL than decoy receptors on the cell surface; whereas fibroblasts isolated from autoimmune disease rheumatoid arthritis expressed both decoy receptors. While the role of the decoy receptor DcR2 in controlling the TRAIL-induced apoptosis remains unknown, further studies are required to reveal the relationship between the pathology of keloid formation and the up regulation of DcR2. Nevertheless, it opens a novel research possibility in understanding the apoptotic behaviors of the pathologic cells.

2.4.5 Down-regulations of TNFRSF11B in Keloid-derived Fibroblasts

TNFRSF11B is more commonly known as OPG, or osteoprotegrin, which means the bone protector. It is another member of the tumor necrosis factor receptor superfamily

and is a soluble protein secreted by the cell (Yasuda et al, 1998; Tsuda et al, 1997). It was first discovered by Simonet et al in 1997, and its role against bone resorption has been quickly revealed.

RANK (Receptor activator of nuclear factor κ B) is a transmembrane receptor, which is also known as TRANCE Receptor, primarily secreted by dendritic cells and skeletal tissue such as bone and skeletal muscle (Anderson et al, 1997; Nakagawa et al, 1998). Once activated, it can stimulate osteoclast transformation from its precursor cell to the mature osteoclast (Takahashi et al, 1999; Fuller et al, 1999). RANK ligand, also known as TRANCE, is a signal transducer of the RANK pathway and it is shown to be able to mediate osteoclastgenesis and hence bone resorption (Yasuda et al, 1998). OPG on the other hand, can compete with RANK and interrupt the cell to cell signal transduction, and hence inhibiting osteoclast maturation and activation, which in turn protect the bone from resorption.(Simonet et al 1997; Tsuda et al, 1997)

Interestingly, the RANK-RANKL interaction is also closely related to our immune system. Besides skeletal tissues, RANKL is also highly up-regulated in activated T-cells (Wong et al, 1997); while RANK is most commonly seen in dendritic cells (Anderson et al, 1997) – cells in our immune system responsible for capturing and presenting antigens

to T-cell, and hence activating our immune response. In normal circumstances, dendritic cells have to be quickly removed by apoptosis after interacting with T-cells, via FAS or TRAIL pathway (Ingulli et al, 1997). Failure in the apoptotic regulations will lead to excessive immune response or even autoimmune diseases. Wong et al (1997) were the first to show that interaction between RANKL on activated T-cells and RANK on dendritics cells promoted dendritic cells survival. Research by Josien et al (2004) also showed that RANKL treatment enhanced the persistence of mature antigen-presenting dentritic cells and increased the antigen-specific T-cell response.

OPG, the secreted and soluble decoy receptor for RANKL is speculated to be able to interfere with the RANK-RANKL interaction and hence down-regulating the immune response by removing the protection signals on dendritic cells (Leibbrandt et al, 2008). Ashcroft et al in 2003 demonstrated that OPG can reduce dendritic cells survival and hence down-regulated T-cell mediated inflammation in IL-2 deficient mouse with autoimmunity.

Our group has, for the first time, reported a down-regulation of OPG in keloid-derived fibroblasts when compared with normal fibroblasts. Given the important role of OPG in modulating the inflammatory response, particularly by reducing the survival of dendritic

cells, we speculate that the down-regulation of OPG may play a role in the pathology of the keloid disease.

The keloid scar is characterized by a prolonged and over-reactive inflammatory response towards injury. A localized decrease in OPG secreted by keloid scar fibroblasts may contribute to the prolonged inflammation. Further studies are required to investigate the role of OPG on the localized immune response of keloid patients and the keloid scar formation.

2.4.6 Up-regulations of TNFRSF12A in Keloid-derived Fibroblasts

TNFRSF12A, also known as Fn14, is by far the smallest member of the tumor necrosis factor superfamily identified. It has also the shortest cytoplasmic domain of the entire family consisting of merely 28 amino acids (Han et al, 2003; Wiley et al, 2001). The only known ligand that interacts with Fn14 is TWEAK (Han et al, 2003). TWEAK was first discovered by Chicheportiche et al in 1997, together with its small receptor Fn14 which lacks a functional cytoplasmic domain. They were considered a weak apoptosis pathway and did not deserve much attention.

However, instead of inducing apoptosis, Tweak-Fn14 is now shown to play an important role in modulating tissue regeneration after injury (Girgenrath et al, 2006; Jakubowski et al, 2005). It is also believed to be related to various tissue pathologies associated with inflammatory diseases (Chambell et al, 2004). Over-expression of TWEAK and its receptor Fn14 is able to amplify the inflammatory response. Abnormal expression of Fn14 has been observed in autoimmune disease, or even cancers. (Chambell et al, 2004; Burkly et al, 2007)

TWEAK-Fn14 in tissue injuries

TWEAK is a soluble cytokine primarily secreted by most inflammatory cell types including monocytes, macrophages, dendritic cells and activated T-cells *etc*(Maecker et al, 2005; Burly et al, 2007; Wiley and Winkles, 2003). Fn14 on the other hand, is hardly detectable in normal cells under normal situation. However, its expression was shown to be increased quickly following tissue injury (Feng et al, 1999; Zheng and Burkly,2008) . Interestingly, Fn14 expressions can only be found in cells under the epithelial, mesenchymal and endothelial lineages that altogether formed the building blocks of our body tissue (Girgenrath et al, 2006; Meighan-Mantha et al, 1999). However, its expression was not seen in T and B cells (Maecker et al, 2005). Therefore, the

Tweak-Fn14 pathway is often thought to be a pathway how the systemic immune system exerts its control over the functional tissues.

In 2005, Jakubowski et al demonstrated that TWEAK is capable of simulating the proliferation of hepatic precursor cells after liver injury, hence suggesting the TWEAK-Fn14 pathway's important role on regeneration after tissue injury.

In 2006, Girgenrath et al has induced injury by injecting cardiotoxin into Fn14-deficient mice and found that the inflammatory response was greatly reduced and delayed. The group has determined that Fn14 expression is crucial for the proliferation of the muscle pro-genitor cells, which is essential for tissue repair after cardio-injury. Therefore, down-regulation of Fn14 directly leads to deficiencies in the expansion of myoblasts and results in an unsuccessful wound healing response.

Soon after tissue injury, the onset of wound healing response will lead to infiltration of inflammatory cells to the damaged site and the release of growth factors such as PDGF and FGF to promote tissue repair. These signals were shown to be able to trigger Fn14 expressions in the TWEAK responsive cells of various types of tissues, including the joint (synoviocytes and chondrocytes), the kidney (mesangial and glomerular/tubular epithelial cells), the skin (keratinocytes and fibroblasts), and the nervous systems (astrocytes and Schwann cells) (Kumar et al, 2009; Chorianopoulos et al, 2009; Burkly,

2007; Girgenrath et al, 2006). With a high level of Fn14 expression, these cells will hence respond to TWEAK mediated proliferation initiated by inflammatory cells for tissue repair. When the inflammation response subsides, the expression of both TWEAK and Fn14 will return to their basal level when robust tissue regeneration is no longer required. However, in some pathological conditions, prolonged over-expression of Fn14 has been observed. The effect of the high level expression has been studied in many pathological conditions such as fibrous degenerative diseases, autoimmune diseases, and cancer (Jakubowsji et al, 2005; Kamijo et al, 2008; Kumar et al, 2009).

Tweak-Fn14 in pathological inflammations

Many wound healing disorders and inflammatory diseases often come with a self-sustained over-reactive inflammatory response localized within the target tissues. Studies have suggested that persistent elevated expression of TWEAK/Fn14 may contribute to the pathology. It may act as the effector molecule that links the disorder in inflammatory cells to specific tissues.

Jakubowski et al in 2005 has observed an up-regulated expression level of Fn14 in liver cirrhosis, a pathological condition where normal hepatic tissue is replaced by fibrous scar tissue. The expression was found in bile ducts and the fibrotic regions and was significantly up-regulated when compared with normal liver. Furthermore, Jakubowski's group also observed that Fn14 is capable of inducing the proliferation of oval cells (precursor for liver cells). Over-expression of Fn14 in transgenic mice resulted in hyperplasia in liver. Cirrhosis was first identified as the result of abnormal collagen synthesis by fibroblasts in liver. However, the linkage between the abnormal behavior of fibroblasts as the effector cells and the inflammatory cells was not known in the past (McGee & Fallon, 1978). The observation of an elevated expression level of Fn14 in liver may suggest its role in wound healing disorders and abnormal formation of fibrous scar tissues.

Rheumatoid arthritis, an auto-immune disease, is characterized by over-proliferation of synovial fibroblasts that have a tendency to invade into cartilage and bone, forming fibrous scar tissue and affecting the movements and functions of the joints. Kamijo et al in 2008 has studied the role of TWEAK-Fn14 in the pathology of Rheumatoid arthritis. The group has detected Fn14 expression in RA synovial fibroblasts and reported that

cultured RA synovial fibroblasts have up-regulated proliferation stimulated by TWEAK. Anti-TWEAK and anti-Fn14 on the other hand, was shown to be able to suppress cell proliferation and cytokine production. The group concluded that TWEAK-Fn14 may contribute to the pathology of RA by stimulating the proliferation of the synovial fibroblasts and up-regulating the expression of pro-inflammatory cytokines. The role of TWEAK on promoting inflammation particularly acting on fibroblasts was further confirmed by Kumar et al in 2009. The group studied the biological response of fibroblasts towards TWEAK and obtained that it is able to induce proinflammatory signaling pathways through activation of TGF-beta-activated kinase 1.

To conclude, current studies on the TWEAK/Fn14 pathway reveal its important role in wound healing. TWEAK-Fn14 is now thought to be able to amplify local inflammatory response. Its actions also include expansion of tissue cells after tissue injury and the modulation of the wound healing response. Persistent up-regulation of Fn14 on tissue cells, particularly fibroblasts, is observed within the affected sites of some fibrous degenerative diseases and auto-immune diseases. Evidence shows that the pathway may be involved in local pathologic expansion of fibroblasts and over-production of collagen leading to accumulation of fibrous connective tissues at the diseased sites.

In our study we have for the first time observed nearly a 3-fold up-regulation of Fn14 in keloid-derived fibroblasts, while the expression in normal fibroblasts was only minimal. This observation was in line with current studies that Fn14 should be only minimally expressed by normal tissue cells under normal situation. Given the existing studies on the action of TWEAK-Fn14, we propose that over-expression of Fn14 in keloid-derived fibroblasts could possibility play a role in the pathology of keloid. Similar to the case of cirrhosis and rheumatoid arthritis derived fibroblasts, keloid fibroblasts are shown to have over-proliferation and over-production of collagen, which directly leads to the excessive scarring. The Fn14 over-expression was shown to have exerted its effect on the mentioned diseases as previously discussed. It would be very interesting to investigate whether the same logic could extend to the keloid and hence possibly provide a brand new perspective to the pathology of the mysterious keloid scar. Furthermore, it could even be made a therapeutic target and provides new solutions to keloid treatment.

2.4.7 Up-regulations of TNFRSF6B in Keloid-derived Fibroblasts

TNFRSF6B, more commonly known as DCR3, is a relatively new member of the tumor necrosis factor receptor (TNFR) superfamily, and was first discovered by Pitti et al in 1998. As reported by Pitti, the protein apparently lacked a transmembrane sequence and therefore was thought to be a soluble, secreted protein rather than a membrane associated molecule. The group also showed that DCR3 binded to FasL and inhibited the FasL activity. Yu et al (1999) discovered that DCR3 was also able to suppress LIGHT-mediated apoptosis by binding to LIGHT in addition to FasL. DCR3 mRNA was over expressed in malignant tissue such as in lung and colon cancer (Pitti et al, 1998; Bai et al, 2000).

The linkage between over-expression of DcR3 and some pathological conditions has been investigated recently. In 2006, Hayashi et al was the first group to investigate the expression of DcR3 in synovial fibroblasts derived from patients with rheumatoid arthritis. The group speculated that as synovial fibroblasts derived from rheumatoid arthritis showed reduced apoptosis and proliferate aggressively as tumor cells, DcR3 may play a role in the pathologies. In This group was the first to identify the expression of DcR3 in rheumatoid arthritis synovial fibroblasts. They have treated rheumatoid arthritis synovial fibroblasts with recombinant human FasL and found that they were resistant to Fas-mediated apoptosis. They also disrupted the expression of DcR3 in

rheumatoid arthritis synovial fibroblasts using DcR3 small interfering RNA. The results showed that the resistance to Fas-mediated apoptosis was greatly reduced. The group concluded that the expression of DcR3 may be one of the reasons for reduced apoptosis in synovial fibroblasts derived from rheumatoid arthritis.

As discussed earlier, fibroblasts derived from keloids showed altered apoptosis regulation, causing a proliferation- apoptosis imbalance and may ultimately leading to the excessive scarring. Lu et al (2007), Chodon et al (2000) and Ladin et al (1998) all observed that Keloid-derived fibroblasts showed resistance to apoptosis *in vitro*. Chodon et al (2000) observed that while keloid-derived fibroblasts showed significant resistances to Fas mediated apoptosis, the Fas receptor expressions appeared to be the same as control.

It is interesting to compare the behavior of the synovial fibroblasts isolated from rheumatoid arthritis and dermal fibroblasts isolated from keloid scarring. RA derived fibroblasts in vitro showed increased proliferation and reduced apoptosis (Kusunoki et al, 2005; Liagre et al, 2007). Clinically, RA fibroblasts have the tendency to invade into normal tissue, replace the functional synovial cartilage into fibrous tissue (Guiducci et al, 2005). All these observations are significantly similar to keloid fibroblasts which also

showed increased proliferation, causing increase in fibroblast population in active keloid scars. In addition to the excessive growth, keloid-derived fibroblasts also showed the tendency to invade into normal tissue, over-producing collagen resulting in a disrupted skin organization and excessive scarring. Although RA and Keloid scarring are two very different kinds of diseases, it is striking for the similarities shared between the phenotypic behaviors of fibroblasts.

In our studies we have observed a significant up-regulation of DcR3 mRNA expression in keloid-derived fibroblasts when compared to normal fibroblasts. Although the action of such an increase is not yet investigated in our study, it is reasonable to speculate that the role of DcR3 in keloid may be similar to that of rheumatoid arthritis given the shared similarities of fibroblasts between the two diseases. Similar to rheumatoid arthritis, DcR3 may be one of the reasons why keloid-derived fibroblasts exhibit resistance to Fas-mediated apoptosis (Lu et al, 2007). In our microarray results, there is no difference in expression levels of Fas receptor in keloid and normal skin. This also agrees with Chodon's findings in 2000, that keloid's resistance to Fas-mediated apoptosis did not come from the difference in expression of Fas receptors. Some molecules may exist to inhibit the Fas pathway. Being a decoy receptor of Fas, localized increase in DcR3 expressed by keloid-derived fibroblasts may compete with Fas receptor for Fas ligand,

and hence protect the fibroblasts from undergoing apoptosis and leads to the excessive scarring. Further studies are required to confirm the observation.

2.5 Summary

In this part of study we have identified 5 apoptosis-related genes expressed differently in keloid and normal fibroblasts, amongst, OPG, Fn14 and DcR3 have crucial roles in regulating the wound healing and immune responses. This may suggest that in order to study the pathogenesis of keloid, it may be useful to look at the defects in the scar's healing response through the secretion of cytokines to recruit or eliminate tissue cells.

Early in 1979, Cohen et al has studied the systemic and localized immune parameters in 45 keloid patients. The group demonstrated that there was significantly increased local immune response in keloid with an increased level of immune complex observed locally in the keloid lesion. However, systemic immune parameters have shown no difference between keloid patients and 200 controls. Kazeem in 1988 also observed elevated levels of immune complexes in keloid and suggested that alterations in the immune response may contribute to the scar formation. In 1990, Martin & Muir observed increased number of inflammatory cells infiltrating in keloid tissue when compared to normal. Ten

years later, Boyce et al (2000) also observed increased number of macrophages in between the collagen bundles in keloid scars. There were also significantly more T cells observed in keloid with a significantly higher CD4 : CD8 ratio. However, despite the attempts to address the immunological aspects associated with keloid formation, little has been known about the relationship between these observations and the pathogenesis of keloid. Keloids are often referred to as the result of an "over exuberant" healing response. However, despite the many years of effort, the drivers of the abnormal healing response have remained unknown.

In our studies we have observed a significant down-regulation of OPG in keloid-derived fibroblasts. In the immune system, OPG is known to be able to attenuate RANK-RANKL interaction and hence down-regulate the immune response by helping to eliminate cells that produce pro-inflammatory cytokines. The down-regulation of OPG observed in keloid may possibly lead to the accumulation of active pro-inflammatory cells locally within the scar, and prolong the local inflammation process. This agrees with the observations in the literature that there is an increase in the number of inflammatory cells found in keloid scars. Over proliferation of fibroblasts and over production of collagen may hence be the consequence of the down-regulation of OPG secreted locally by fibroblasts.

The up-regulation of Fn14 in keloid-derived fibroblasts may provide another pathway for the excessive development of scar tissue as discussed earlier. Fn14 mainly acts on fibroblasts by letting them respond to pro-inflammation cytokines released by cells from the immune system. Over expression of Fn14 was shown to lead to over production of fibrous tissue and prolonged inflammation. On the other hand, up-regulation of DcR3 by keloid-derived fibroblasts may protect the cells from apoptotic signals and hence leads to the proliferation-elimination imbalance.

2.6 Conclusions

In our studies, we have identified the up-regulation of several interesting genes in keloid-derived fibroblasts when compared with normal skin fibroblasts using MicroArray analysis. The result is confirmed with real time-PCR. All these findings in our study are novel and they open new opportunities in the studies of keloid pathogenesis. We speculate the possibility of the interaction between an altered immune response in the lesion and the altered apoptosis regulations in keloid fibroblasts. We here

provide a different perspective in keloid pathology and open many opportunities for potential future studies.

2.7 Further research plans

The differential expression of OPG, DcR3 and Fn14 at the protein level by keloid and normal fibroblasts needs to be confirmed using western blotting analysis. Furthermore, the role of DcR3 on keloid scarring can be studied in vitro by blocking the DcR3 expression in keloid-derived fibroblasts and examine their susceptibility to apoptosis. In addition, it is also interesting to look at the expression of DcR3 *in vivo* by immunohistochemcial staining in keloid scar biopsies to confirm its clinical significance as a therapeutic target.

On the other hand, Fn14 has been reported as a therapeutic target to suppress inflammation in some inflammatory diseases such as liver cirrhosis and rheumatoid arthritis (Jakubowski et al, 2005; Kamijo et al, 2008). Its role in keloid scarring can be further studied by introducing Fn14 antibodies into keloid fibroblast *in vitro* and compare the collagen synthesis, proliferation and apoptosis.

3 Laser Interaction in Keloid Scarring

3.1 Introduction

3.1.1 Laser – an overview

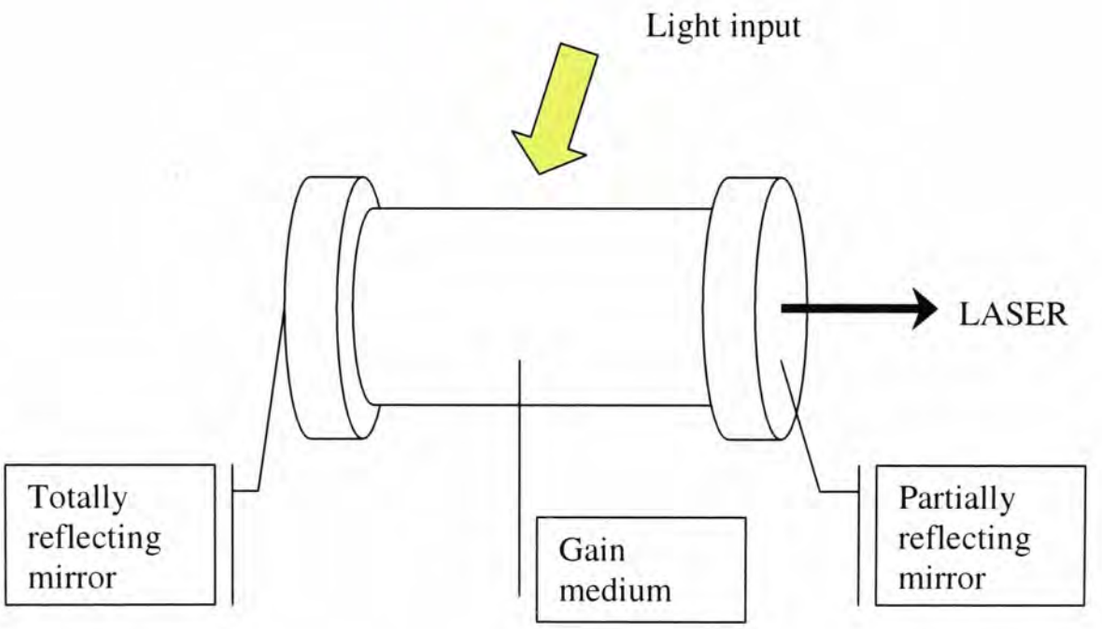


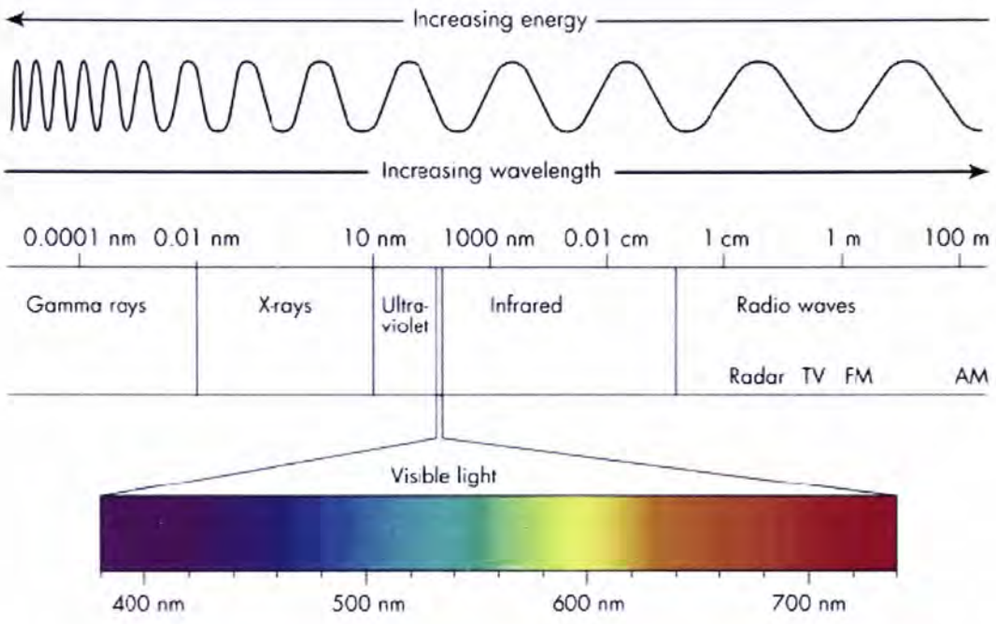
Figure 6. The mechanism of Laser

The term LASER means Light Amplification by the Stimulated Emission of Radiation. Lasers produce a coherent light beam of a specific wavelength. The production of a laser beam involves supplying energy to a cavity containing the gain medium between two mirrors, with one being semi-transparent and acting as the outlet of the laser beam. The gain medium is the material inside a laser machine that can amplify the light that enters the space with a characteristic wavelength when a particle is passing through it. Gain

mediums can be in "solid state" which is a combination of solid crystals, or "gas state", which contains gas molecules. The particles inside a specific gain medium can be stimulated when being "pumped" with energy and some of their electrons are excited to a higher quantum level. The electrons return to the ground state by emission of a characteristic wavelength of light reflecting the change in the energy state, a single wavelength light beam is hence produced. The single wavelength light beam will be reflected back and forth between two mirrors and each time passing through the gain medium, and amplification of light is thus achieved. The polarity of reflected light is also synchronized and the coherent laser light beam escapes through the semi-transparent mirror. The range of visible light is 400 nm-780 nm, which means lasers with wavelengths falling outside this range cannot be seen by the human eye. Lasers have been used clinically for more than 40 years now (Tanzi et al, 2003). Their application in treating abnormal scars started in the mid-1980s. Common types of lasers are summarized as follows:

Gain Medium	Type of laser	Wavelength	Spectrum
Argon	gas state	488/514nm	blue-green
Nd:YAG	solid state	1064/532nm	infrared/ green
Organic Dye	liquid state	585-595nm	yellow
Er:YAG	solid state	2490nm	infrared
Carbon Dioxide	gas state	10600nm	infrared

Table 2. Different types of laser with different gain mediums



http://www.antonine-education.co.uk/physics_gcse/Unit_1/Topic_5/em_spectrum.jpg

Figure 7. The light spectrum

3.1.2 Biological Effects of Laser on Skin

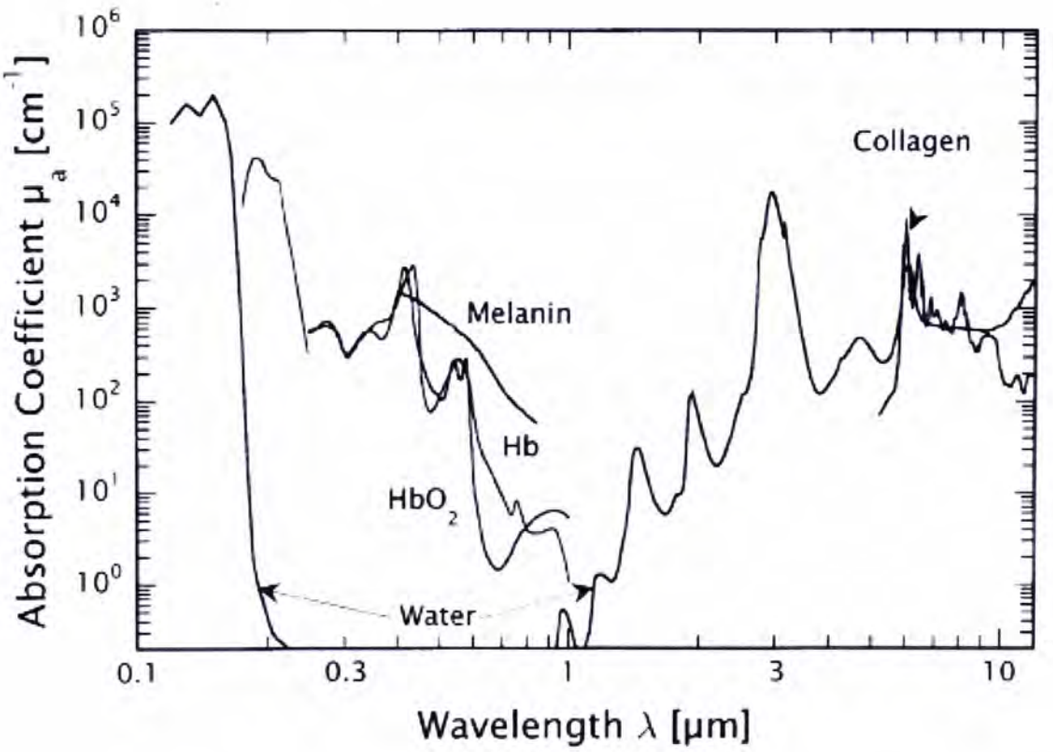
Tissues consist of cells embedded in an extracelluar matrix (ECM). Measured by weight, the extracellular matrix mainly consists of water and collagen (Vogel & Venugopalan, 2003). Some molecules are also present in smaller amount such as elastin, glycosaminoglycans, glycoproteins and cell adhesion proteins together to give the characteristic physical and biological properties of the ECM (Silver, 1987).

In human skin, collagen accounts for approximately 30 % of the total weight with water accounting for nearly all the rest (Craig, 1975). Therefore, studies on laser-collagen and laser-water interaction will give a picture of laser-matrix interaction (Vogel & Venugopalan, 2003).

3.1.3 Laser-matrix interaction

The laser matrix interaction is summarized in a book written by Niemz in 2004. When laser energy is delivered to skin, there will be a rise in temperature and resulting in a gain in kinetic energy of collagen fibrils. Denaturation of collagen refers to the situation when collagen has gained enough kinetic energy to overcome the forces that keep the fibers in place, and the intact helical structure relaxes, hence losing mechanical strength. The maximum temperature the collagen in skin can tolerate depends on the number of crosslinks present in the collagen molecules (Niemz, 2004). Generally, older people have more crosslinks in skin, and therefore, can tolerate a higher energy than younger individuals when exposed to laser (Niemz, 2004). Note that collagen denaturation not only depends on the thermal effect introduced by a particular type of laser, but also the pulse duration of laser. A shorter pulse of laser, the higher the maximum temperature collagen can tolerate (Caroll & Humphreys, 2006).

Light of different wavelength will be differently absorbed by different molecules in skin. The ability of different molecules to absorb energy of a particular wavelength is measured as the absorption coefficient. The absorption coefficients of water, hemoglobin, melanin and collagen are summarized by Vogel and Venugopalan (2003) in the following figure.



Vogel and Venugopalan, 2003

Figure 8. The absorption coefficients of different tissue components

As shown in this figure, outside the visible range (wavelength = 400 - 780 nm), light energy is mainly absorbed by water, and collagen; while inside the visible range, energy

is mainly absorb by melanin, oxy-hemoglobin and deoxy-hemoglobin. Unlike UV light, which can be largely absorbed by DNA in the cell and can directly cause DNA fragmentation and potentially genetic alternation, visible range laser mainly asserts its effect through a thermal effect. The depth of penetration of laser into skin depends on the wavelength, as different wavelength will have absorption peaks for different elements. Longer wavelength (like Nd:YAG 1064 nm) laser is poorly absorbed by water, and other pigments (hemoglobin, melanin) in skin, and therefore it has highest penetration into skin (Vogel & Venugopalan, 2003). Research done by Goh in 2003 showed the penetration depth of Nd:YAG 1064 nm laser is 6 - 7 mm.

Laser energy in the visible range, such as 585 nm PDL and 532 nm frequency doubled Nd:YAG laser is greatly absorbed by hemoglobin and melanin in skin, and therefore only have a penetration depth of less than 0.7mm (Hohenleutner et al, 1996). It is widely hypothesized that the action of 585 nm PDL on hypertrophic scar is by selectively delivering energy to the microvessels which damages the blood supply to the highly proliferative hypertrophic scar and hence forcing it to regress (Bowes et al, 2002; Parrett & Donelan,2009).

3.1.4 Laser-cell interaction

Unlike the laser-matrix interaction, the laser-cell interaction is less well understood, and it is believed to be more complex than the laser-matrix interaction which only involves biophysical phenomenon. The effect of laser on cells depends heavily on the wavelength and the pulse duration. For example, red laser (around 700 nm) is equally absorbed in all sub-cellular regions of a cell and therefore can damage the whole cell unselectively (Sheetz, 1998). Shorter wavelength blue green laser (around 600 - 500 nm), on the other hand, is absorbed selectively by some sub-cellular regions and therefore can be used with more specific biological functions (Sheetz, 1998). Laser pulses with longer pulse duration will greatly increase the temperature surrounding the cell. Significant temperature rise will kill the cell directly via thermal effect (Caroll & Humphreys, 2006). With shorter pulse duration (e.g. in nanoseconds), the environment surrounding the cell can dissipate the heat very quickly (Keller et al, 2001). Temperature rise will hence be localized in sub-cellular regions where the absorption is high (Sheetz, 1998)

Laser energy mainly interacts with cells mainly via two different mechanisms: firstly by creating shock waves on cell membranes and hence altering its structure (Sheetz, 1998); and secondly via natural chromophores such as the respiratory organelles (Vogel &

Venugopalan, 2003; Sheetz, 1998). A single cell experiment on exposure of pulsed Nd: YAG 532 nm demonstrated that the laser can create a transient alteration in electrical activity of the outer membrane of the cell and make the membrane more permeable to exogenous molecules (Kitzes & Berns, 1979). Ziegler and Chiu in 2009, and Shelby et al in 2005 have demonstrated that laser can also alter cell viability mainly via the disruption of the plasma membrane, and can lead to necrosis or apoptosis depending on the radiation mechanism and energy.

3.1.5 Clinical Use of Laser in treating Keloids

Lasers have been used clinically to treat keloid and hypertrophic scars for more than twenty years. The following paragraphs summarize the timeline for the use of several lasers on scars and their efficacy.

The 1980s

The use of laser in treating keloid scars started in the mid 1980s. Castro et al (1983) were the first ones to observe a decreased collagen production in normal fibroblasts after 1064 nm Nd:YAG laser treatment, and later started the trend of research of laser effect

on abnormal scars. Apfelberg et al (1984) was the first group to study the use of the argon laser and carbon dioxide laser excision of earlobe keloids of 13 patients. In their study, however, only 1 patient responded to the treatment while the remaining 12 showed no improvements. Abergel et al (1984) treated keloid fibroblasts cultures with Nd:YAG 1064 nm laser and observed decrease in collagen production. In their clinical studies 8 patients with keloid lesions were treated with nondestructive laser dosage and after 3 years of follow up, the scars were flattened and softened.

Hulsbergen-Henning et al (1986) used "disappointing" to conclude their results in a clinical study involving 45 patients with keloid scars using Argon laser. Only 3 showed positive results while 28 showed poor results in the study. The group observed shrinkage of keloid immediately after treatment but the scar recurred several days after. This result however, supported Apfelberg's poor results with the Argon laser on 1984.

The carbon dioxide laser's effects on keloids are not promising either. In 1989, Stern and Lucente used the carbon dioxide laser to excise 23 earlobe keloids and 17 recurred after the surgery, and the group concluded that carbon dioxide laser excision failed to lower the recurrence rate of keloids. 5 years after the initial failure of treating earlobe keloid with carbon dioxide and Argon, Apfelberg et al tried the CO_2 laser excision again

in 1989 and obtained equally disappointing results. 8 out of 9 keloids in earlobe, back or trunk region recurred 10 - 22 months after treatment, and Apfelberg concluded the use of CO_2 laser on keloid scar excision as failure. Norris (1991) also reported similar results with CO_2 laser excision and concluded the modality failed.

To conclude, the use of Argon laser in treating keloid scars and keloid excision using CO_2 is not satisfactory. Nd:YAG 1064 nm seemed to have some positive effect on keloid scars; however, its effect has not been studied fully. From the mid 1990s, scientists had turned their attentions to another type of laser- the 585 nm pulsed dye lasers (PDL). The effects of PDL on hypertrophic scars were particularly promising and this laser is widely used clinically nowadays. However, the effect on keloid scars is not so predictable.

.

<u>The 1990s</u>

In 1995 Alster et al treated median sternotomy keloid scars with 585 nm PDL. The treated group showed improved texture. In 2000, Connell and Harland treated 10 patients with recalcitrant keloid scars with 585 nm PDL followed by intra-lesional steroid and the combined effect was reported as satisfactory. When earlier results

appeared promising, Paquet et al (2001) tried to improve the appearance of keloid using 585 nm laser and found that there were no significant effect on erythema of keloid. Manuskiatti and Fitzpatrick in 2002 revealed that there were no significant differences in treatment outcome between 585 nm PDL and intralesional steroid treatment; however, 585 nm PDL was relatively free from adverse effects. Kuo et al (2004) recruited 30 patients with keloid and treated them with 585 nm laser at intervals of 2 months with mean fluence of 14 J/cm^2. Result revealed that 26 patients showed greater than 50 % regression of keloid after twelve months. The group also observed that more treatment received under regular time intervals would yield better treatment outcome.

Unlike the positive outcome in treating hypertrophic scars, there are no agreements on the efficacy of laser on keloid scars. The discrepancies in results can be due to the lack of large scale clinical trials and the lack of quantitative and comparable outcome measures. Also, the wealth of parameters that are involved in the laser treatment complicates the evaluation of results. Even for the same type of laser used, different spot size and intensity can result in very different outcomes. It is generally observed that higher energy will yield better result than lower energy. But if the energy is too high it will increase the risk of side-effects (Bouzari et al, 2007).

Nd: YAG 532 nm Laser

The Nd:YAG 532 nm laser is widely used clinically in tattoo removal and treatment of pigmented lesions due to its high absorption coefficient by the pigment melanin in skin. The wavelength of Nd:YAG 532 nm laser is close to 585 nm PDL, and therefore, some studies have compared the effects of 532 nm and 585 nm on scars. Bowes et al (2002) recruited 10 patients with hypertrophic scars and divide each scar into areas of equal size. One of these areas was treated with 585 nm PDL, 10mm spot size, 3.5 J/cm^2 and pulse duration of 450 μs; one treated with frequency doubled QS Nd:YAG 532 nm laser, 3 mm spot size, 2.8 J/cm^2 with pulse duration of 10 ns; and one was left untreated acting as control. The results showed significant improvement of all treatment groups when compared to control. But there were no significant difference in treatment outcome between 585 nm PDL and QS 532 nm laser. The group concluded that QS Nd:YAG 532 nm laser is at least as effective as 585 nm PDL in treating hypertrophic scars.

The clinical effects of 585 nm laser on scars have been relatively well studied. However, there are few studies designed to study the effect of 532 nm laser on keloid scars. Therefore, studying the effect of 532 nm laser on such scars becomes important as it may potentially provide further strategies to treat this clinical challenge. Also, the

mechanisms of how laser exerts the biological effects on the cells are still yet to be fully discovered. Previous studies by our group (Poon et al, 2005) have evaluated the effect of QS Nd:YAG 532 nm laser on normal fibroblasts monolayer culture using a sub-lethal dosage. Our group has obtained the viability of normal fibroblasts under different fluence of laser exposure, and determined the sub-lethal dosage on monolayer normal fibroblasts culture. Our group observed the increase in SCF, HGF and b-FGF gene expression in fibroblasts under repeated sub-lethal laser exposure by RT-PCR analysis. In our previous study, although the other biological effects of Nd:YAG 532 nm laser on keloid fibroblasts were not specifically examined, it clearly revealed the ability of the laser to affect cell behavior by regulating gene expression.

Therefore, it would be of interest to explore the effect of the laser with higher intensity in order to reveal a better picture of Nd:YAG 532 nm laser's potential in treating keloid scarring.

3.1.6 Methods of Laser Research on Keloids

In order to study the laser-cell interaction for clinical applications, experimental models have to be developed to mimic the biological environment of the cells of the skin. Many

different models have been developed to serve this purpose. The most common ones are being 1) monolayer cell model 2) 3D tissue engineered model and 3) tissue biopsies.

The monolayer cell model

This is the first, simplest and most direct model in evaluating the results of laser on fibroblasts. Cells were seeded in culture dishes or wells. Medium was removed just before laser radiation and was replaced quickly afterwards. In this way, the cells were exposed to laser directly free of the interference with other factors. The results were clear and could easily be evaluated quantitatively. Cells after treatment could be easily obtained for viability or proliferation experiments. Protein secretion could also be analyzed through the collection of the conditional culture medium. Previous studies by our group (Poon et al, 2005) on the effect of Nd:YAG 532 nm laser have used this monolayer cell model. However, monolayer cultures may be over-simplified the complex biological environment of cells. Results obtained thus may not be of direct clinical relevance.

The 3D tissue engineered model

Keloid scarring is a unique condition in humans and cannot be found in any other animals. Therefore keloid research is particularly challenging because of the lack of animal models. Using a 3D tissue engineered model may be useful for the studies on laser-tissue interactions without involving as significant ethical problems as taking biopsies from keloid patients. In 2005, Chiu et al constructed a raft that involves mixing fibroblasts in a collagen matrix with keratinocytes seeding on the top for laser experiments. Normal and keloid fibroblasts and keratinocytes were chosen for the construct, and the effect of photodynamic therapy was studied. Photodynamic therapy involved introducing a photosensitizing agent (5-amino levulinic acid) which will absorb the laser energy and enhancing the laser effect. The viability of cells after laser treatment was evaluated using confocal microscopy. The group observed that the viability of keloid fibroblasts was higher than that of normal fibroblasts.

This 3-D tissue engineered model is a sophisticated model in laser research that simulates the natural architecture of the skin and is of potential clinical relevance. However, more than one type of cells was involved in this model and there were far more variables to control than that in monolayer cell cultures. For example, the collagen density and the cell density of fibroblasts and keratinocytes have to be carefully controlled in each sample. Qualitative examination of results may be more useful for this

model such as histological staining. However, quantitative biological parameters such as protein expression are more difficult to evaluate in this model.

Tissue biopsies after laser treatment

Following positive clinical results of 585 nm PDL treatment on keloid in 2004, Kuo et al performed studies on the mechanism of keloid regression induction by 585 nm PDL. In 2005, the group obtained punch biopsies of keloid scars from 10 patients before and 7 days after laser treatment (mean fluence equal to 14 J/cm^2 as in their previous clinical study). They then looked at the proliferation of keloid cells by PCNA, the apoptosis by TUNEL array and cleaved caspase-3, and MAP kinase activation by p38, ERK and JNK expressions using immuno-histochemical staining. The group reported a decrease in PCNA-positive cells after laser treatment, an increase in TUNEL-positive cells and a moderate increase in caspase-3 activation. Expression of ERK and p38 was also increased; however, the level of JNK stayed more or less the same. The group concluded that 585 nm laser suppressed keloid formation by suppressing proliferation and enhancing apoptosis of the fibroblasts.

Obtaining punch biopsies directly at the site of treatment would be the best way to reveal the laser effect on tissue. Besides identifying the expression levels of proteins of interest by immuno-histochemical staining, the coagulation depth reached by the laser and the subsequent extracellular matrix remodeling would be shown clearly. However, histochemical studies are qualitative measurement of treatment outcome. The number of positive cells can be counted under microscope and compared with different treatment groups. But the total number of cell was not known. Therefore, we do not know what percentage of cells have undergone apoptosis. Also, due to the limited punch biopsies researchers can obtain from patients, the time response of cells on laser cannot be studied in detail. The group did not provide reasons for choosing 7 days post treatment as the time for evaluation of treatment effect. However, their studies still provide useful information for the studies on laser tissue interactions.

It may be also worth pointing out that punch biopsies introduce wounds to the keloid patients, and may have the risk of inducing further keloid scarring, there may be some ethical issues involved in such studies. Obtaining patients consent to participate in such studies may also be particularly difficult.

To conclude, there are a number of methods developed for studying the laser cell interactions, each with their advantages and disadvantages in the experimental designs. As a first step to study the biological effect of Nd:YAG 532 nm laser on keloid-derived fibroblasts, it is preferable to start with the simple and direct monolayer cell model so that the results are clear and easily measurable. If a positive result is observed, more sophisticated models such as the 3D tissue engineered model or even tissue biopsies could be used to obtain more concrete results for clinical applications.

3.2 Material and method

3.2.1 Cell Culture

Fibroblasts culture was obtained as described previously in section 2.2.1

3.2.2 Laser radiation

Keloid scar fibroblasts were seeded on 6-well plates at 1 X 10^5 cells per well and cultured in DMEM + 10 % FBS until 80 % confluence. Culture medium was then removed and cells were washed twice with PBS prior to laser exposure at energy 0.55 and 1.0 J/cm^2 in duplicates.

The laser machine used in this study was Versapulse Cosmetic Laser System (coherent medical group). The laser was a Q-switched frequency doubled Nd:YAG 532 nm laser with pulse width of 4 ns. The machine could deliver a maximum energy of 200 mJ through a handpiece with adjustable spot sizes of 2,3,4,5 and 6 mm in diameter at frequency 1, 2 4 and 10 Hz or single shot. The handpiece was fixed using a clamp and the cell culture in a 6-well plate was put on top of a rotor right beneath the handpiece. The 6-well plate was placed in an inverted position to ensure direct exposure of the cells. A red piece of cardboard was placed underneath the 6-well plate to reduce reflection.

DMEM + 10 % FBS was added to each well immediately after laser treatment.

3.2.3 Trypan blue exclusion

Two keloid fibroblast cell lines were used in the experiment in triplicate. At 24 and 48 hours after laser treatment, cell viability was measured using trypan blue exclusion method. The cells were collected by trypsinization using 0.5 mL 0.25 % trypsin – 1mM EDTA (Gibco BRL) for 3 minutes. 1 µl of the cells were stained with 1 µl of 0.4 % trypan blue (Sigma) solution. The number of viable (not stained) and dead (stained) cell were counted and presented as the percentage over total number of cells. Statistical significance was tested using 2-sided T-test against control with n=6.

3.2.4 Annexin-V flow cytometry

Two keloid fibroblast cell lines were used in the experiment in triplicate. At 24 and 48 hours after laser treatment, the cells were harvested using 0.25% trypsin-EDTA (Gibco BRL) and washed twice with PBS. Floating cells that detached from cultures vessels during this period of culture after laser exposure were also collected by centrifuging the culture medium. Combined cell pools were finally resuspended in Annexin-V-Fluos

labeling solution.

Diluted labeling solution was prepared by mixing 40uL Annexin-V-Fluos labeling regent, 40 µl Propidium iodide solution in 2 ml Incubation buffer (Annexin-V-Fluos staining kit, Roche). 250 µl of the labeling solution was used to resuspend the washed cell pellet and was incubated for 15 minutes in room temperature. The cells were then analyzed on a flow cytometer. Statistical significance was tested using 2-sided T-test against control with n=6.

3.2.5 BrdU cell proliferation assay

Two keloid fibroblasts cell lines were used in the experiment. At 24 and 48 hours after laser treatment, cell proliferation activity was measured by cell proliferation ELISA, BrdU kit (Roche Molecular Biochemicals). 100 µl BrdU labeling reagent was added to each well and incubated for 4 hours in humidified CO_2 incubator at 37 °C. Attached cells were fixed with Denfix solution for 30 min and then incubated with anti-BrdU antibody for 2 hours at room temperature. After washing three times with PBS, chromogen-containing substrate was developed for 30 min and the reaction was terminated by the addition of 500 µl 1M H_2SO_4. The chemiluminescence was measured

at 450 nm using 690 nm as a reference. Results were expressed as the relative cell proliferation activity compared to sham-irradiated controls. Statistical significance was tested using 2-sided T-test against control with n=2.

3.3 Results

3.3.1 Cell Viability of Keloid Fibroblasts After 532 nm Laser Treatment

Under the laser condition at 3 mm/s rotor speed, 5 mm spot size and 1 Hz pulse frequency, fibroblasts in 6 well plates were exposed under 0, 0.55 and 1.0 J/cm^2 laser. Trypan blue exclusion was performed for the assessment of cell viability, flow cytometry of Annexin V/ PI for apoptosis and BrdU assay for cell proliferation were performed in triplicates for 2 keloid fibroblasts cell lines. The results are shown as follows.

Cell Viability by Tryphan Blue Exclusion

	Control	0.55J/cm2	1.0J/cm2
24 hours	93.00%	91.98%	86.86%
SD	2.51%	2.13%	4.52%
n	6	6	6
p		**0.46**	**0.05 ***
48 hours	93.48%	94.49%	71.78%
SD	1.45%	0.54%	13.09%
n	6	6	6
p		**0.16**	**0.01 ***

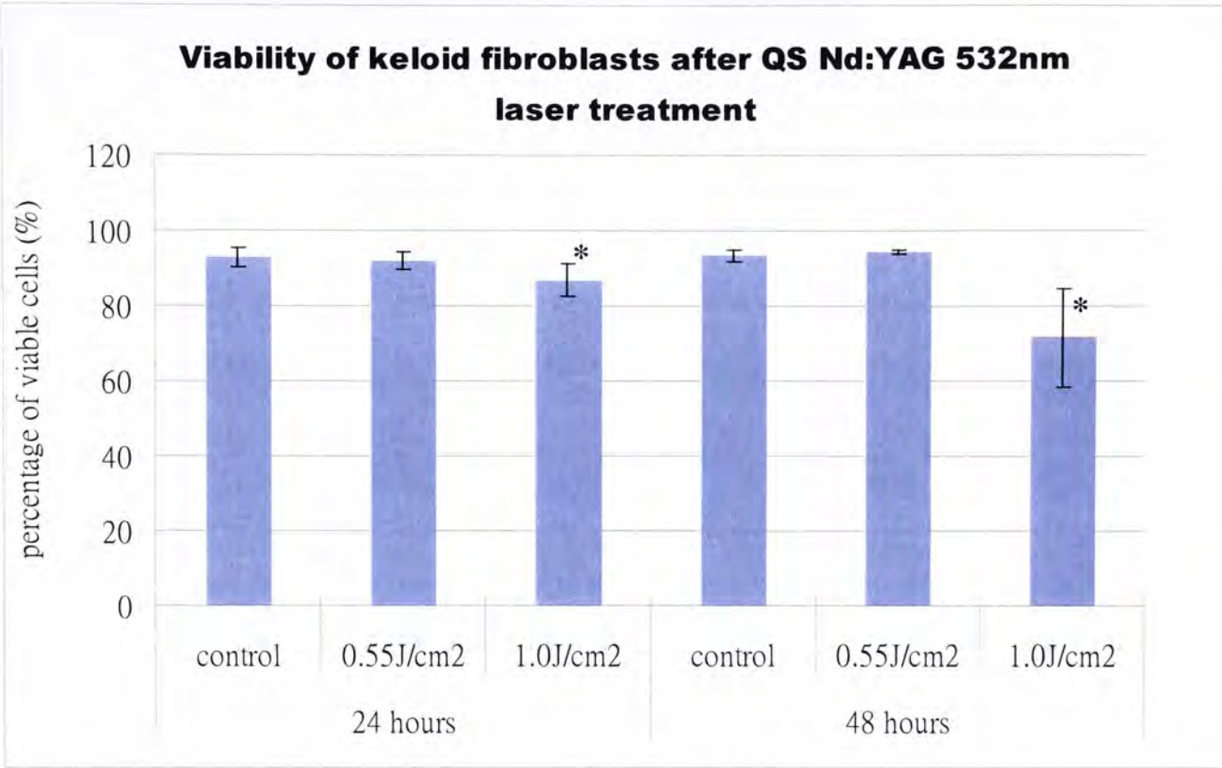


Figure 9. Viability of keloid fibroblasts after laser treatment

By using trypan blue exclusion, the viability of keloid fibroblasts of all groups at 24 hours was above 85 %, with the control and 0.55 J/cm^2 treated group greater than 90 %. At 48 hours, the control and 0.55 J/cm^2 treated group still maintained viability greater than 90 %, while the viability of 1.0 J/ cm^2 dropped sharply to around 70 %. Under 2-sided T-test, the difference between 1.0J/ cm^2 treatment group and the control at both 24 and 48 hours has statistical significance.

3.3.2 Apoptosis VS Necrosis In Percentage Of Cell Death

Percentage of Apoptotic Cells

	Control	0.55J/cm2	1.0J/cm2
24 hours	3.46%	4.19%	7.67%
SD	1.31%	1.37%	2.69%
n	6	6	6
p		0.11	**0.01 ***
48 hours	2.81%	2.83%	18.26%
SD	1.06%	0.16%	9.03%
n	6	6	6
p		0.96	**0.01 ***

Percentage of Necrotic Cells

	Control	0.55J/cm2	1.0J/cm2
24 hours	3.42%	3.05%	5.05%
SD	1.36%	2.13%	4.52%
n	6	6	6
p		0.58	**0.11**
48 hours	3.60%	2.59%	9.70%
SD	1.45%	0.57%	3.96%
n	6	6	6
p		0.02 *	**0.01 ***

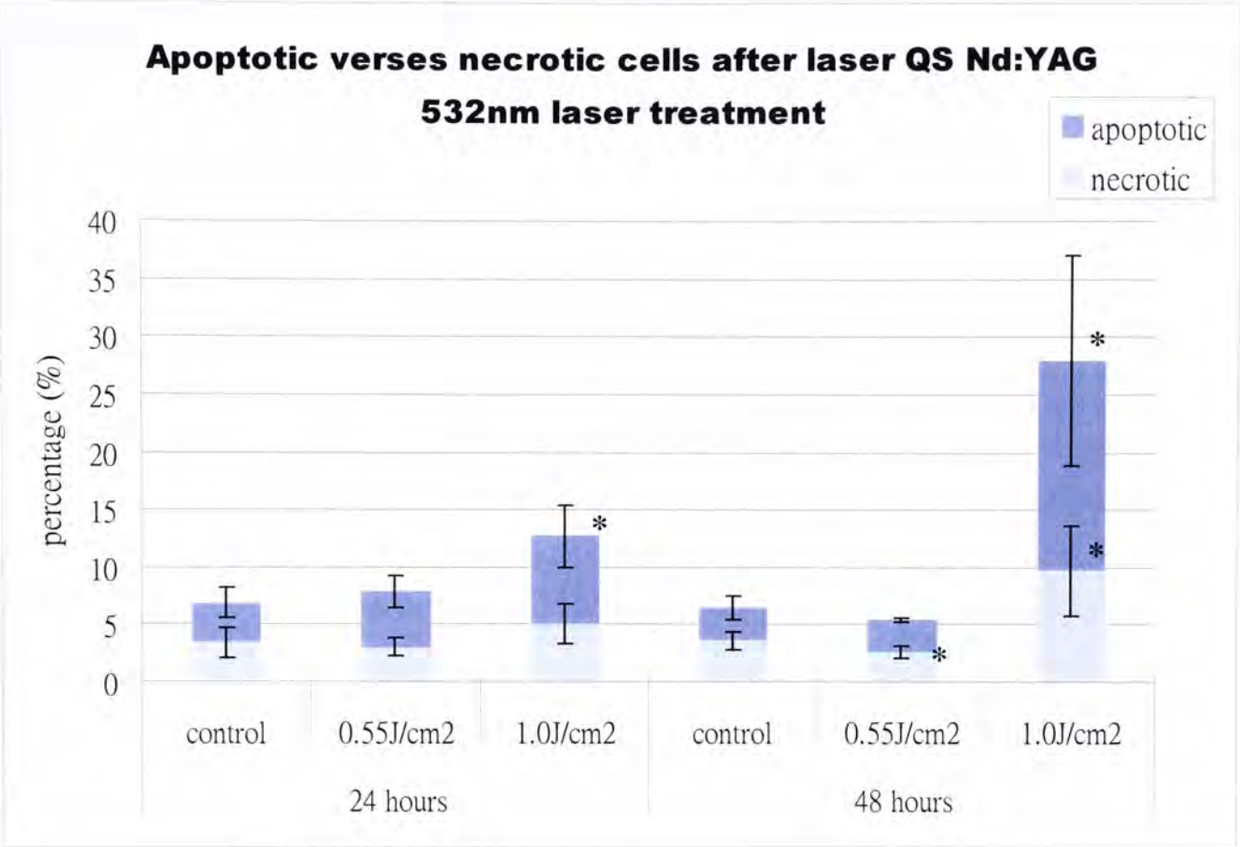


Figure 10. Percentage of apoptotic and necrotic cells after laser treatment

For apoptotic verses necrotic cells, at 24 hours, less than 5 % of cells were undergoing apoptosis for the control and 0.55 J/cm^2 treated group; while for 1.0 J/cm^2 treated group, there was around 7 % of apoptotic cells, under 2-sided T-test, the difference was statistically significant. At 48 hours, the percentage of apoptotic cells was around 3 % for the control and the 0.55 J/cm^2 treated group, while for 1.0 J/cm^2 treated group, around 18 % of keloid fibroblasts were apoptotic, the difference was statistically significant. Also, for the 1.0 J/cm^2 treated group, the percentage of necrotic cells was 9 %, only half of the apoptotic cells.

3.3.3 Cell proliferation after 532 nm laser treatment

Cell Proliferation by BrdU assay

	Control	0.55J/cm2	1.0J/cm2
24 hours	1	1.4	0.62
SD		0.38	0.33
n	2	2	2
p		**0.38**	**0.39**
48 hours	1	0.76	0.74
SD		0.24	0.34
n	2	2	2
p		**0.37**	**0.48**

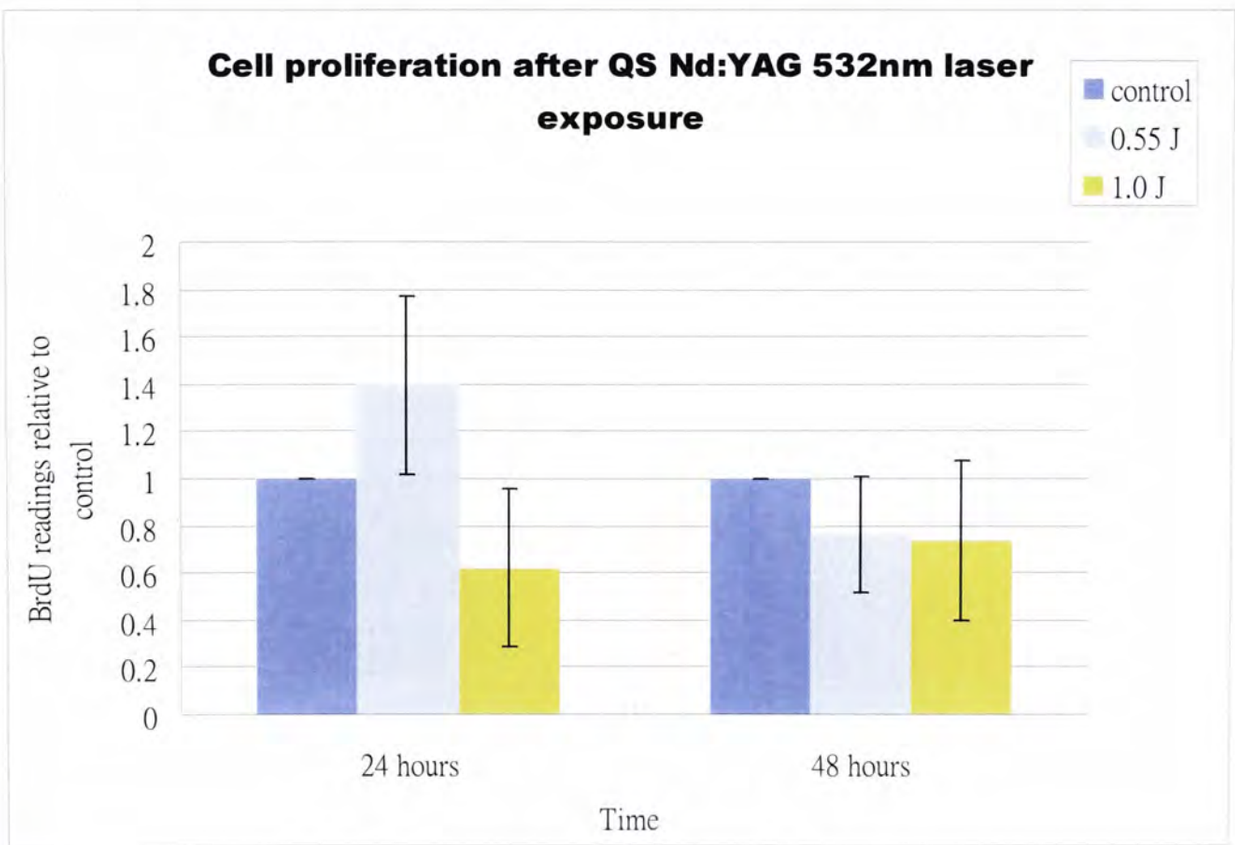


Figure 11. Cell proliferation after laser treatment

The above BrdU readings were normalized by dividing the treatment groups readings over the control group reading. At 24 hours, the proliferation rate for the 0.55 J/cm^2 treatment group was 1.4 fold as compared to the control whereas the 1.0 J/cm^2 treatment group was only 60 % of the control. At 48 hours, the cell proliferation in both 0.55 J/cm^2 and 1.0 J/cm^2 treatment groups dropped slightly as compared to the control.

3.4 Discussions

3.4.1 Discussions on Optimization of Laser Parameters

The laser machine used in this study is Q-switched frequency doubled Nd:YAG laser at 532 nm, with pulse width 4 ns, from Versapulse® Cosmetic Laser System (Coherent medical group). The diameter of the laser pulse is adjustable at 2, 3, 4, 5 and 6 mm, delivering total energy ranging from 100 mJ to 200 mJ per pulse. Under this system, given the same total energy, smaller the spot size, higher the energy density of each laser pulse. For example, a 5 mm spot with energy density equals to 1.0 J/cm^2, and a 4 mm spot with an energy density equals 1.5 J/cm^2, will deliver the same total energy of 200 mJ per pulse.

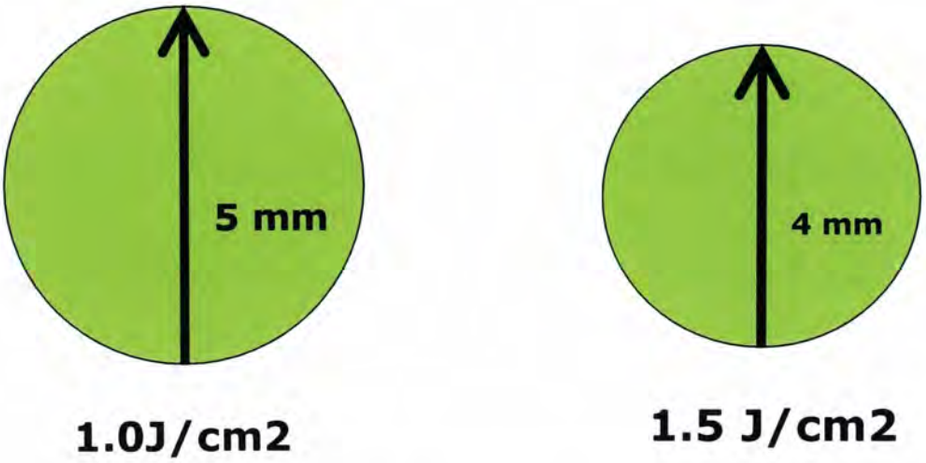


Figure 12. Relationship between spot size and laser energy density

In our experiment, the laser probe of the Versapulse laser machine is fixed above a rotor using a clamp. The culture plate is placed on top of the rotor that can move at a constant

speed of 3 mm/s in a sequential motion row by row. The rotor will advance by 3 mm at the end of each row.

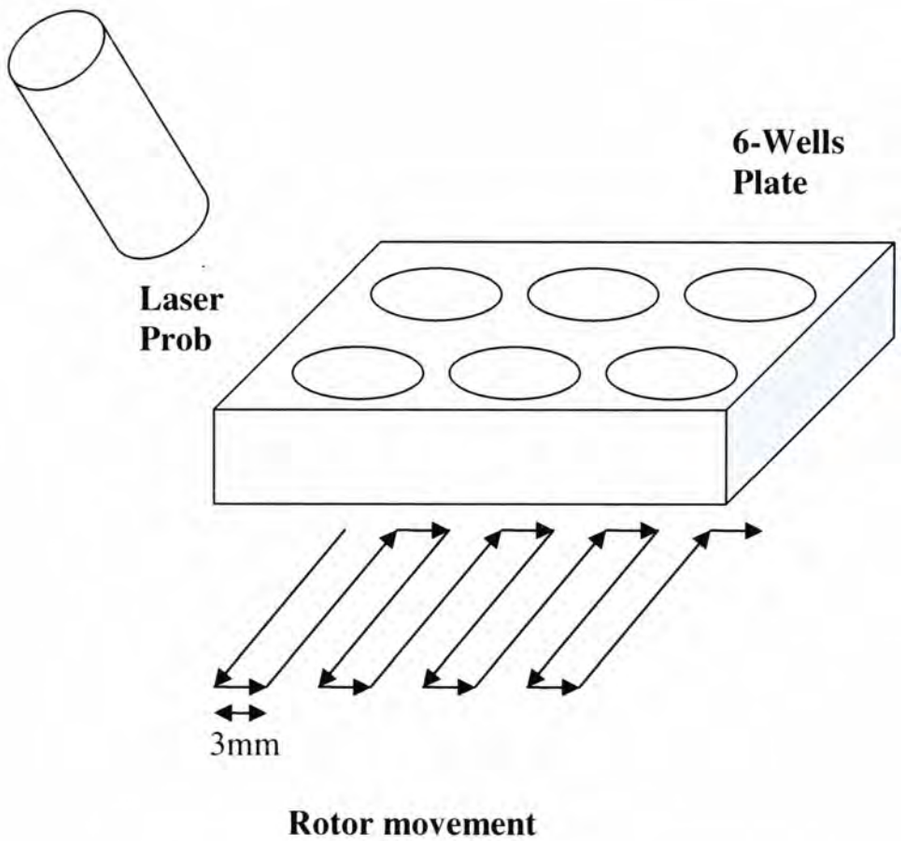


Figure 13. The experimental set up of laser treatment

The pattern of the delivery of laser pulses depends on the frequency of pulse delivery (number of pulse delivered per second) and the spot size. If a laser pulse of 3 mm in diameter is delivered at a frequency 0.5 Hz, there will be a pulse every 6 mm and the spots would be too far apart, with 3 mm of space in between two pulses being unexposed. If the frequency was too high (say 10 Hz), there will be overlapping of laser pulses and the cells would be over-exposed to the laser energy.

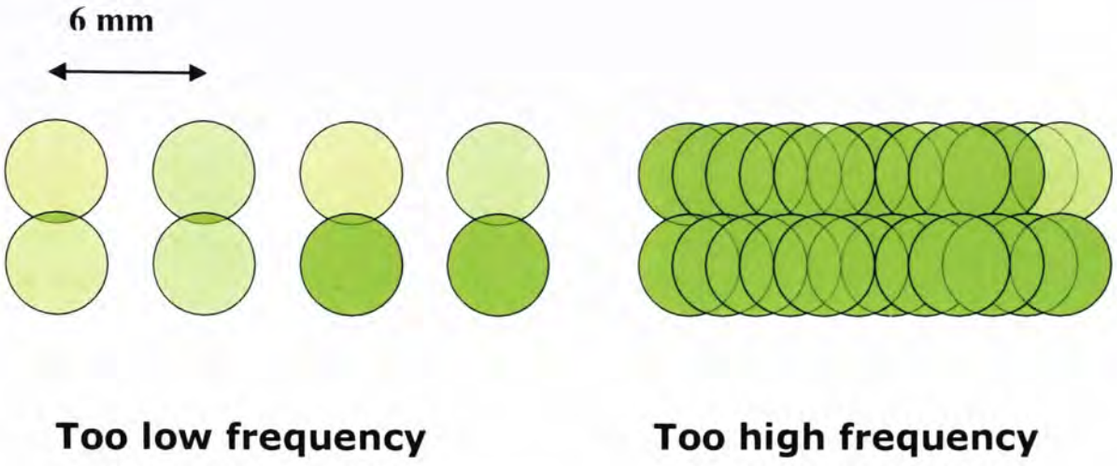


Figure 14. The relationship of laser shots frequency and cell exposure

An even coverage of laser radiation to the cells is the key to obtain a repeatable and measureable result. Following careful calculation, the 5 mm spot size and the 1 Hz pulse delivery were chosen as the optimal laser parameter. The laser pulses were slightly overlapped in comparable amount both vertically and horizontally. Each cell is exposed to laser without too much overlapping. Several other spot size and frequencies were tested in this study, the 5 mm spot size with 1 Hz pulse frequency was observed to give the most consist results.

Figure 15 The optimal laser parameters

Another point to note was that it was ideal to perform the experiment under one single spot size because when the spot size was changed, the frequency also needed to be changed, and the overlapping areas would not be the same. Therefore, results obtained

under different spot sizes might not be comparable. Using the 5 mm spot size, the maximum and minimum energy density could be achieved was 0.55 to 1.0 J/cm^2. As established in our previous study by Poon et al in 2005, 0.8 J/cm^2 was the cutting line for sub-lethal and lethal dosage on normal fibroblasts after QS Nd:YAG 532 nm laser treatment for 24 hours using MTT. Increasing the energy density beyond 0.8 J/cm^2 would yield a sharp decrease in viability while below 0.8 J/cm^2 the cell viability was high. The range 0.55 - 1.0 J/cm^2 offered by the 5 mm spot size well covered the lethal and sub-lethal transition region and hence would give more information of cell behavior under this range.

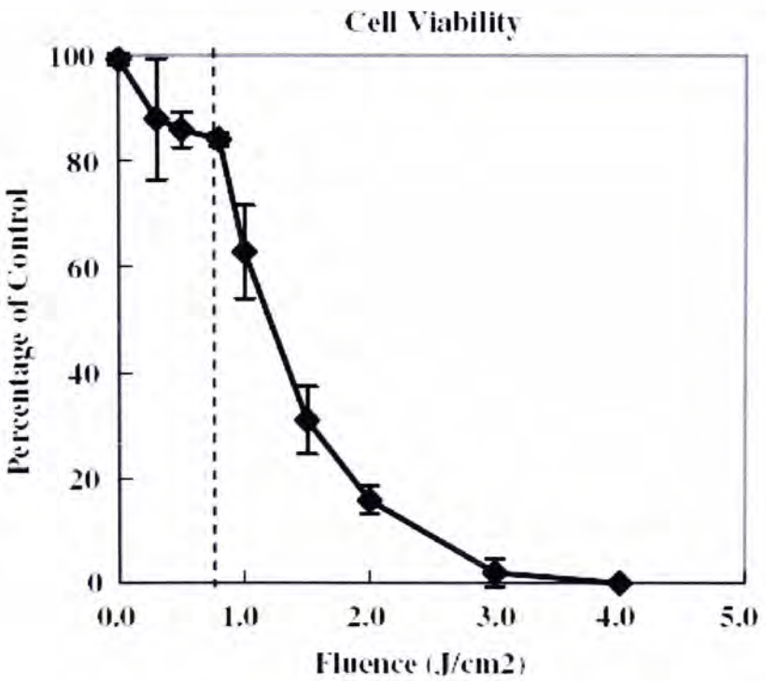


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Figure 16. Cell viability under different laser energy density

3.4.2 Discussion on the Biological effects of Nd:YAG 532 nm laser

3.4.2.1 Decreased viability in the 1.0 J/cm^2 treatment group at 48 hours

In our studies keloid-derived fibroblasts were treated with QS Nd:YAG 532 nm laser of two energy levels: the sublethal 0.55 J/cm^2 dosage and the lethal 1.0 J/cm^2 dosage. As shown in our cell viability results, the 1.0 J/cm^2 treatment group showed the lowest percentage of viability at both 24 and 48 hours. While the viability of the 0.55 J/cm^2 treatment group was not different from that of the control, the viability of the 1.0 J/cm^2 treatment group at 24 hours is 87 %, implying that 13 % of keloid fibroblasts were kill within the first 24 hours after laser treatment. Interestingly, at 48 hours, the viability further dropped to 72 %, meaning that a further 15 % of keloid-derived fibroblasts died within 24 to 48 hours after laser treatment. Ziegler and Chiu in 2009 observed that cell death via necrosis following laser radiation should occur immediately after the treatment (within 24 hours). Therefore, the delay in cell death occurred at 48 hours after treatment may not be contributed by necrosis alone.

3.4.2.2 Increased apoptosis in the 1.0 J/cm^2 treatment group

The ability of Nd:YAG 532 nm laser to induce apoptosis on keloid fibroblasts could be revealed more clearly by the flow cytometry using Annexin V vs PI. Both the 0.55 J/cm^2 treatment group and the control group showed apoptotic cell of around 3 % - 4% of total cell number at both 24 hours and 48 hours after treatment. The percentage of necrotic cells was also around 3%.

For the 1.0 J/cm^2 treatment group, at 24 hours, the percentage of necrotic cells was around 5 %, meaning only less than 2 % more keloid fibroblasts were killed by the laser under necrosis at 1.0 J/cm^2 when compared with control. However, at 48 hours, a striking 18 % of cells were apoptotic in the 1.0 J/cm^2 treatment group ($p=0.01$). The results also revealed the time course of apoptosis following laser treatment. Between 24 hours to 48 hours, around 10 % more cells were undergoing apoptosis. This observation agreed with the literature that while necrosis is an immediate process following cell damage, cell death via apoptosis would take longer than 24 hours (Ziegler and Chiu, 2009).

This was a fascinating observation because keloid scarring is believed to be related to an over-reactive wound healing response for a prolonged period of time leading to the excessive scarring. Any treatments that can introduce cell death by necrosis, for instance

surgical removal, will have the risk of stimulating the inflammation response and lead to further scarring. Apoptosis on the other hand, is a quiet way of cell elimination without involving the simulation of the wound healing cascade. Utilizing the apoptosis pathway to eliminate keloid fibroblasts would be one of the ideal ways of treating Keloids. In this study we determined that Nd:YAG 532 nm laser is capable of inducing a significant portion of keloid fibroblasts to undergo apoptosis ($p=0.01$), with the necrotic cells portion relatively small. This indicated the great potential of laser to be developed into an effective treatment strategy for keloid scarring.

3.4.2.3 Increased proliferation in the 0.55 J/cm^2 treatment group

The BrdU assay on cell proliferation had revealed an interesting effect of the 0.55 J/cm^2 laser. At 24 hours, while the proliferation of the 1.0 J/cm^2 treatment group was only half of the control group, the 0.55 J/cm^2 treatment group showed 40 % more proliferation than the control. This may possibly suggest that lower dosage of 532 nm Nd:YAG laser was capable of inducing cell proliferation. Also, at 48 hours, the flow cytometry result also showed a significantly lower percentage of necrotic cells in the 0.55 J/cm^2 treatment group when compared with control. This provided very useful information on the clinical use of the laser. Practitioners have to be careful in choosing a sufficient dosage

for treating keloid. If the dosage is too low, the laser may induce proliferation of the keloid fibroblasts and may potentially worsen the situation.

3.5 Summary and conclusion

As discussed in chapter 2, Keloid scarring is a very challenging clinical situation. The recurrence problem is particularly difficult to deal with. Many treatment strategies have been developed but each of them has their own benefits and drawbacks.

The fibroblasts derived from keloid lesions demonstrate reduced apoptosis. This is believed to be one of the reasons for the formation of keloid scars due to the ineffective elimination of cells. However, treatment methods that look to eliminating the cells and

the excessive scar tissue by creating wounds will activate the inflammatory response and can potentially stimulate further scar formation.

Treatment strategies targeted to induce apoptosis in keloid may be effective in stimulating keloid regression, and may reduce the chance of recurrence. The currently used treatment strategies based on induction of apoptosis is the electron radiation. Unlike laser, which is a neutral light beam; electron radiation is highly ionizing and can cause considerable damage to the DNA of the cells. Electron radiation can be effective on inducing apoptosis in keloid: however, concerns are whether such radiation will eventually lead to malignancy.

The use of Nd: YAG 532 nm laser is an emerging treatment modality for keloid scarring and has been shown to be effective in treating keloid scars clinically. It is a relatively safe method with minimal side effects when compared to other treatment strategies. However, the biological mechanisms on keloid fibroblasts have not been established yet in the literature. In this study, we have observed that Nd: YAG 532 nm laser was capable of inducing apoptosis in keloid-derived fibroblasts. More importantly, a relatively insignificant percentage of cell were necrotic under laser treatment, meaning that the laser can induce apoptosis within a keloid lesion and triggers minimal

inflammatory response. It can potentially develop into a treatment modality with low chance of recurrence.

The most effective energy level of Nd:YAG 532 nm to be used in treating keloid fibroblasts was observed to be 1.0 J/cm^2 in our study. Although the energy level received will be different in monolayer cell culture and the skin, it provides useful information that by optimizing the treatment parameter, the laser can deliver its best treatment results. It is also worth noting that when the laser is used clinically, it would be essential to make sure the dosage used was adequate in order to prevent stimulation of keloid proliferation.

3.6 Further research plans

In this study, the Nd:YAG 532 nm laser was demonstrated to be effective in inducing apoptosis in keloid-derived fibroblasts in a monolayer cell culture model. This model is simple and straight forward however the relevance to clinical conditions remains uncertain.

Therefore, more sophisticated models such as 3D tissue-engineered models may be used to reveal more information on the biological effects of the laser.

In chapter 2, the differential expression of apoptotic genes in keloid fibroblasts versus normal fibroblasts has been demonstrated, and their roles in the keloid pathology have been discussed. Whether laser irradiation can alter the expression levels of the five apoptosis-related genes identified in keloid fibroblasts needs to be further elucidated.

4 Conclusions

In this study, the expression profile of apoptosis-related genes in keloid scar and normal skin-derived fibroblasts was examined using microarrays. Five apoptosis-related genes were identified to be differently expressed. OPG, Fn14 and DcR3 have been reported to play important roles in the regulation of wound healing and immune response. This finding not only opens new opportunities for the study of keloid pathology, it may also provide a different perspective in the pathogenesis of keloid scarring which may help the development of some new treatment modalities.

Laser treatment is one of the emerging treatment methods for keloid scarring. It is relatively safe and has minimal side effects as compared to other treatment strategies. The biological effects of the Nd:YAG 532nm laser, including proliferation, apoptosis and necrosis, on keloid fibroblasts have been investigated in this study. It is observed that the Nd: YAG 532 nm laser was capable of inducing apoptosis in keloid-derived fibroblasts. Of note the percentage of cells undergoing necrosis after the laser irradiation was relatively small, indicating that this laser may be capable of inducing apoptotic instead of necrotic cell death in keloid lesions, and hence triggering only minimal

inflammatory response. Thus it may be developed as a treatment modality with low chance of recurrence of keloid.

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